PLANTAIN (*Plantago lanceolata* L.) AS A NATURAL MITIGATION STRATEGY TO REDUCE NITROGEN LOSSES FROM PASTURE-BASED DAIRY SYSTEMS.

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ABSTRACT

The incorporation of plantain *(Plantago lanceolata*) into cows' diet can reduce urine nitrogen (N) concentrations and modify the soil N cycle in urine patches by inhibiting nitrification. The objective of this thesis was to assess the potential of plantain as a mitigation option for N loss from dairy farms to the environment by measuring N leaching (nitrate [NO_3^-]), and N emissions (nitrous oxide [N_2O] and ammonia [NH_3]) from plantain swards on a Pallic soil, and to evaluate the inhibitor effect of aucubin, a secondary metabolite, on N losses from soil.

An important aspect of this study was the evaluation of NO_3^- leaching at the paddock scale from three pasture treatments (plantain, plantain-clovers mix and ryegrass-white clover) throughout two grazing seasons. Pasture treatments were grazed with lactating cows approximately monthly from September 2017 to May 2019. All the drainage from the grazed plots was monitored and sampled for NO_3^- and total N analyses during the 2017, 2018 and 2019 drainage seasons. The effect of plantain on N_2O and NH_3 emissions was evaluated in two field experiments carried out in spring 2017 and autumn/winter 2018. The N_2O emissions from soil were measured using the static chamber method, and NH_3 losses with the dynamic chamber method. In this field experiment the effects of adding two urine types (from cows grazing plantain or ryegrass-white clover) to two swards (plantain or ryegrass-white clover) were evaluated. The ability of aucubin to reduce N losses from urine patches was studied in a lysimeter experiment where aucubin was applied along with urine from cows grazing ryegrass-white clover pasture to ryegrass-white clover and plantain swards. In a hydroponic experiment, the release of secondary metabolites from a plantain root system was quantified.

The plantain pasture treatment reduced NO_3^- leaching by 48 and 58% (2.6 and 3.8 kg NO_3^--N ha^{-1}, respectively) compared to ryegrass-white clover and plantain-clovers mix pasture treatments, in the 2018 drainage season. During the 2019 drainage season, NO_3^- leaching between the plantain (4.0 kg NO_3^--N ha^{-1}) and ryegrass-white clover (3.1 kg NO_3^--N ha^{-1}) treatments were similar, which was likely due to a lower proportion of plantain in the sward during the critical summer-autumn period. In spring 2017, even though the urea-N concentration was 45% lower in urine of cows fed plantain than in urine of cows fed ryegrass-white clover swards, the cumulative N_2O emissions from urine of cows grazing plantain was similar to urine of cows grazing ryegrass-white clover swards. Nitrous oxide emissions were reduced 31% by the plantain sward (1.4 kg N_2O-N ha^{-1}) in spring 2017. In autumn/winter 2018, N_2O emissions from urine of cows grazing plantain (urea-N concentration was 40% lower in the urine of cows fed plantain than in the urine of cows grazing ryegrass-white clover pastures) were 48% lower (4.3 kg N_2O-N ha^{-1}) than from urine of cows grazing ryegrass-white clover. However, in this season, the cumulative N_2O losses were 69% (7.1 kg N_2O-N ha^{-1}) higher from the plantain sward than the ryegrass-white clover sward and were associated with an increase in the water filled pore space (WFPS) in the plantain soil. The lower urea-N concentration in urine from cows fed plantain compared to ryegrass-white clover reduced NH_3 losses from plantain and ryegrass-white clover swards in both spring 2017 and in autumn/winter 2018. The lysimeter experiment also showed that plantain swards reduced N_2O emissions by 50% (9.0 kg N_2O-N ha^{-1}) compared to ryegrass-white clover swards. The addition of aucubin reduced the N_2O emissions from urine patches by 36% in ryegrass-white clover swards. Only small concentrations of secondary metabolites were detected in the roots exudates of plantain plants grown in the hydroponic experiment. A high aucubin concentration (12 g kg^{-1} DM) in plantain leaves was observed after 50 and 60 days of the hydroponic study.

In conclusion, this research demonstrates the potential of plantain to mitigate N leaching from pastoral dairy systems and N_2O and NH_3 emissions from urine patches by reducing the N and urea-N concentrations in cow urine. There was also evidence of a plantain sward effect on N dynamics in the soil, which decreased N_2O emissions, possibly through the release of aucubin into the soil via leaf litter.

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LIST OF ABBREVIATIONS

AMO	Ammonia monooxygenase
AMMO 31	Ammonium sulphate: urea, 31 % N,
AOB	ammonia- oxidizing bacteria
Auc	Aucubin
BNI	Biological nitrification inhibition
CO_2	Carbon dioxide
CP	Crude protein
D.A.T	days after transplantation
DC	Duration-controlled
DCD	Dicyandiamide
DM	Dry matter
EF3	Emission factor
HPLC	High-performance liquid chromatography
IG	Iridoid glycosides
M	Molar weight
MS	Milk solid
N	Nitrogen
$(NH_4)_2SO_4$	Ammonium sulfate
N_2O	Nitrous oxide
NI	Nitrification inhibitors
NH_4^+	Ammonium
NH_3	Ammonia
NO_3^-	Nitrate
OAD	Once a day
PL= pl	Plantain
PLE	Plantain leaf extract
Rg	ryegrass
r/w	Ryegrass-white clover
S	Sward
TN	Total nitrogen
V	Volume

Vm	Molar volume
U	Urine
UNc	UN concentration

1 GENERAL INTRODUCTION

Introduction

Agriculture is one of the principal components of the New Zealand economy and the dairy sector, which is one of the largest exporters, contributed over $ (NZ) 18.1 billon to the economy in 2019 (New Zealand Dairy Statistics, 2019). In the 2018/2019 season, New Zealand dairy farms produced 21.2 billion litres of milk containing 1.88 billion kilograms of milk solids (New Zealand Dairy Statistics, 2019). One of the major environmental concerns of the dairy industry is the impact of their nitrogen (N) footprint on freshwater ecosystems and the atmosphere. Nitrate (NO_3^-) enrichment of water bodies and underground aquifers can stimulate the growth of algae causing eutrophication and at high concentrations is toxic for aquatic life and unsafe for recreation (Ministry for the Environment and Ministry for Primary Industries, 2018). Likewise, high NO_3^- concentrations in potable groundwater can be hazardous to human health. The losses of the greenhouse gas (GHG) nitrous oxide (N_2O), is another important loss of N from dairy farms. The agriculture sector in New Zealand represents 48% of the gross national emissions, and the N_2O emissions from soil in dairy farming equates to 22% of the agricultural emissions (Ministry for the Environment, 2020).

The New Zealand Government has policies and targets that will help improve water quality and reduce GHG emissions, and it aims to achieve these improvements ‘within a generation’ (Ministry for the Environment and Ministry for Primary Industry, 2018). The Essential Freshwater programme, and the National Policy Statement on Freshwater Management (NPS-FM) which preceded it, require dairy farmers to implement substantial actions to reduce the impact of the sector on the aquatic environment. New Zealand is also a signatory to the Kyoto Protocol and has to report on its GHG emissions to the United Nations Framework Convention on Climate Change (UNFCCC). The Government’s recent Zero Carbon Bill aims to reduce all GHG emissions. Carbon dioxide (CO_2) and N_2O must be reduced to net zero by 2050, and methane (CH_4) emissions must reduce by 10% by 2030 and an additional 24-47% by 2050 (Ministry for the Environment, 2019). To achieve these reductions, each farm will be required to estimate their emissions and develop a management plan to reduce these losses.

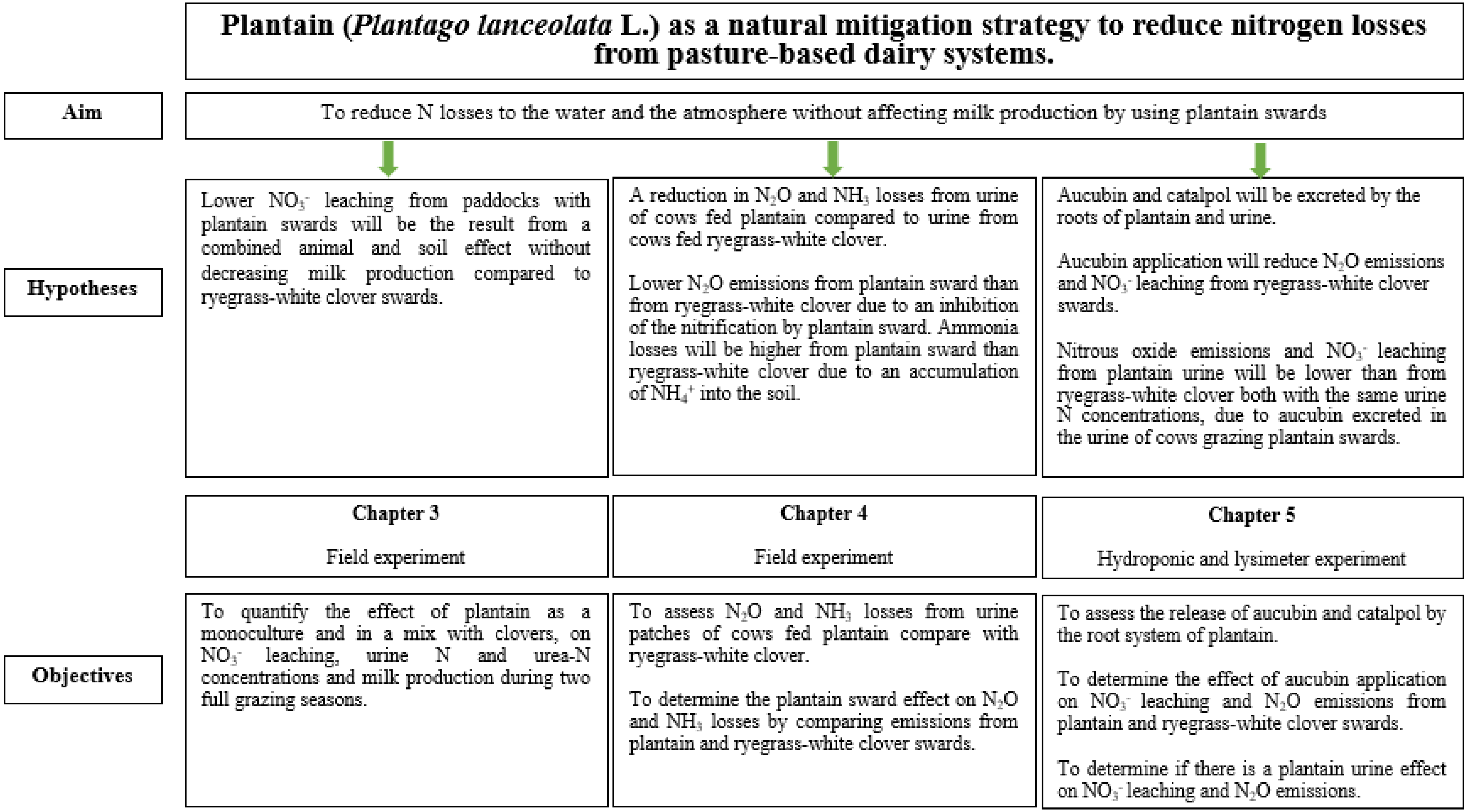
New Zealand’s dairy systems rely on perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture for their cost competitiveness (Charlton & Stewart, 1999; Vibart *et al*., 2016). In these pastoral systems, the N content of the grazed pastures is generally in excess of the N requirements of animals and the surplus N is excreted, mainly in livestock urine (Oenema *et al*., 2005), resulting in very small areas in the soil with N rates up to 1000 kg N ha^{-1} (Di & Cameron, 2002; Haynes & Williams, 1993). The high N rates in these 'hot spots' are well above plant growth requirements, consequently the excess N is at risk of being lost from the soil to the water, as NO_3^- leaching, and/or to the atmosphere, as N_2O emissions which contribute to global warming (IPCC, 2007).

As the main driver of N losses from dairy systems is the N concentration and N loading in dairy cows' urine (Selbie *et al*., 2015), mitigation strategies to reduce N losses to the environment from urine patches have been reviewed by a number of authors (Di & Cameron, 2002, 2016; Luo *et al*., 2010; Saggar *et al*., 2004; Zaman *et al*., 2009). Recently, a promising option to reduce N losses from dairy systems was identified: the use of narrow-leaved plantain (*Plantago lanceolata*). Plantain can decrease the N loading rates (expressed as kg N ha^{-1}) in cow urine and so reduces the N losses from the soil. Short-term studies have found a reduction in urinary N concentration when grazed plantain is incorporated into the cows' diet, compared to an all ryegrass-white clover diet (Box *et al*., 2017; Dodd *et al*., 2018; Mangwe *et al*., 2019; Minnée *et al*., 2017, 2020). Importantly, in these studies, there was no decrease in milk production for the cows fed plantain when it makes up to 30% of their diet.

A several number of studies (Luo *et al*., 2018; Simon *et al*., 2019) have found a reduction in N_2O emissions from grazed plantain swards compared to ryegrass-white clover swards. In addition, compared to ryegrass-white clover pastures, reductions in NO_3^- leaching, ranging from 39 to 89%, have been measured from urine patches in a pure plantain sward or a sward mix containing plantain, compared to ryegrass-white clover (Carlton *et al*., 2018; Welten *et al*., 2019; Woods *et al*., 2018). The reductions in N losses from these experiments have mostly been attributed to the decreases in urine-N concentrations and the excretion of secondary metabolites by the plantain root system. Other studies have reported that aucubin, one of the secondary metabolites, inhibited the soil nitrification process (Dietz *et al*., 2013; Gardiner *et al*., 2019). These findings suggest that plantain has the potential to alter the N cycle by influencing the composition of urine excreted by livestock as well as inhibiting the nitrification process in the soil. However, there is limited information on the effect of plantain swards on N_2O and ammonia (NH_3) losses from poor draining soils. It is also important to quantify NO_3^- leaching at a paddock scale over a full lactation season, which captures the spatial and temporal distribution of urine patches deposited into the paddocks by the grazing cows and the effect of the pasture treatments. The hypotheses and research objectives of this thesis are fully described in Figure 1.1. The main objective of this thesis is to evaluate plantain swards as a natural mitigation strategy to reduce N losses from a Pallic on which the sward is grazed by dairy cows.

Thesis structure

This thesis consists of 6 chapters including a General Introduction (Chapter 1) and a Review of the Literature (Chapter 2). Chapters 3-5 describe the field, glasshouse and lysimeter experiments conducted over the three years of the PhD. Each research chapter has been written as a journal paper, comprising an abstract, introduction, materials and methods, results, discussion, conclusions and references. In Chapter 3, the ability of plantain to reduce NO_3^- leaching from large-scale plots is evaluated in a field trial. In Chapter 4, the direct effect of plantain swards on N_2O and NH_3 emissions are studied as well as the effect of urine from cows grazing plantain on both N_2O and NH_3 losses. In Chapter 5, the release of aucubin and catalpol by plantain root systems is evaluated along with the effect of aucubin on N losses from lysimeters when it is applied to either a plantain or a ryegrass-white clover sward. Chapter 6 focusses on a general discussion of the results obtained in the three research chapters, future research and the main conclusions of the thesis.



Plantain (*Plantago lanceolata* L.) as a natural mitigation strategy to reduce nitrogen losses from pasture-based dairy systems.

Aim

To reduce N losses to the water and the atmosphere without affecting milk production by using plantain swards

Hypotheses	Lower NO_3^- leaching from paddocks with plantain swards will be the result from a combined animal and soil effect without decreasing milk production compared to ryegrass-white clover swards.	A reduction in N_2O and NH_3 losses from urine of cows fed plantain compared to urine from cows fed ryegrass-white clover. Lower N_2O emissions from plantain sward than from ryegrass-white clover due to an inhibition of the nitrification by plantain sward. Ammonia losses will be higher from plantain sward than ryegrass-white clover due to an accumulation of NH_4^+ into the soil.	Aucubin and catalpol will be excreted by the roots of plantain and urine. Aucubin application will reduce N_2O emissions and NO_3^- leaching from ryegrass-white clover swards. Nitrous oxide emissions and NO_3^- leaching from plantain urine will be lower than from ryegrass-white clover both with the same urine N concentrations, due to aucubin excreted in the urine of cows grazing plantain swards.
	Chapter 3 Field experiment	**Chapter 4** Field experiment	**Chapter 5** Hydroponic and lysimeter experiment
Objectives	To quantify the effect of plantain as a monoculture and in a mix with clovers, on NO_3^- leaching, urine N and urea-N concentrations and milk production during two full grazing seasons.	To assess N_2O and NH_3 losses from urine patches of cows fed plantain compare with ryegrass-white clover. To determine the plantain sward effect on N_2O and NH_3 losses by comparing emissions from plantain and ryegrass-white clover swards.	To assess the release of aucubin and catalpol by the root system of plantain. To determine the effect of aucubin application on NO_3^- leaching and N_2O emissions from plantain and ryegrass-white clover swards. To determine if there is a plantain urine effect on NO_3^- leaching and N_2O emissions.

Figure 1.1. The hypotheses, objectives and structure of this thesis.

1.1 REFERENCES

Box, L. A., Edwards, G. R. & Bryant, R. H. (2017). Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research*, *60*(4), 470-482.

Carlton, A. J., Cameron, K. C., Di, H. J., Edwards, G. R. & Clough, T. J. (2018). Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. *New Zealand Journal of Agricultural Research, 62*(2)*,* 150-172.

Charlton, J. F. L. & Stewart, A. V. (1999). Pasture species and cultivars used in New Zealand - a list. *Proceedings of the New Zealand Grassland Association, 61*, 147-166.

Di, H. & Cameron, K. (2016). Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: a review. *Journal of Soils and Sediments, 16*(5), 1401-1420.

Di, H. J. & Cameron, K. C. (2002). The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. *Soil Use and Management, 18*(4), 395-403.

Dietz, M., Machill, S., Hoffmann, H. C. & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil*, *368,* 445-58.

Dodd, M., Dalley, D., Wims, C., Elliott, D. & Griffin, A. (2018). A comparison of temperate pasture species mixtures selected to increase dairy cow production and reduce urinary nitrogen excretion. *New Zealand Journal of Agricultural Research*: *62*(4), 504-527.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019). Efficacy of aucubin as a nitrification inhibitor assessed in two Canterbury field trials. *New Zealand Journal of Agricultural Research*, *63*(1),73-86.

Haynes, R. J. & Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in agronomy*, *49*, 119-99.

IPCC. (2007). Climate Change 2007: The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M., Averyt, K.B., Tignor, M., Miller, H.L. (Eds.). Cambridge University Press, Cambridge, UK and New York, USA, p. 996.

Luo, J., Balvert, S. F., Wise, B., Welten, B., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. & Judge, A. (2018). Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. *Science of The Total Environment*, *610–611,* 1271-80.

Luo, J., de Klein, C. A. M., Ledgard, S. F. & Saggar, S. (2010). Management options to reduce nitrous oxide emissions from intensively grazed pastures: A review. *Agriculture, Ecosystems & Environment, 136*(3–4), 282-291.

Mangwe, M. C., Bryant, R. H., Beck, M. R., Beale, N., Bunt, C. & Gregorini, P. (2019). Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. *Animal Feed Science and Technology*, *252*, 11-22.

Ministry for the Environment and Ministry for Primary Industries. (2018). Essential Freshwater: Healthy Water, Fairly Allocated. Wellington: Ministry for the Environment and Ministry for Primary Industries. ISBN: 978-1-98-852584-6 (online) Publication number: ME 1382.

Ministry for the Environment. (2019). Climate Change Response (Zero Carbon) Amendment Bill: Summary. Wellington: Ministry for the Environment. ISBN: 978-1-98-857926-9. Publication number: ME 1410.

Ministry for the Environment. (2020). New Zealand's Greenhouse Gas Inventory 1990-2018. Fulfilling reporting requirements under the United Nations Framework Convention on Climate Change. Wellington, New Zealand. ISSN: 1179-223X (electronic) Publication number: ME 1496.

Minnée, E. M. K., Leach, C. M. T. & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science, 239,* 104093.

Minnée, E. M. K., Waghorn, G. C., Lee, J. M. & Clark, C. E. F. (2017). Including chicory or plantain in a perennial ryegrass/white clover-based diet of dairy cattle in late lactation: Feed intake, milk production and rumen digestion. *Animal Feed Science and Technology, 227*, 52-61.

New Zealand Dairy Statistics. (2019). https://d1r5hvvxe7dolz.cloudfront.net/media/documents/2018-19_New_Zealand_Dairy_Statistics.pdfOenema, O., Wrage, N., Velthof, G., van

Groenigen, J. W., Dolfing, J. & Kuikman, P. J. (2005). Trends in global nitrous oxide emissions from animal production systems. *Nutrient Cycling in Agroecosystems, 72*(1), 51-65.

Saggar, S., Bolan, N. S., Bhandral, R., Hedley, C. B. & Luo, J. (2004). A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures. *New Zealand Journal of Agricultural Research, 47*(4), 513-544.

Selbie, D. R., Buckthought, L. E. & Shepherd, M. A. (2015). The Challenge of the Urine Patch for Managing Nitrogen in Grazed Pasture Systems. *Advances in agronomy*, *129,* 229-292.

Simon, P. L., de Klein, C. A. M., Worth, W., Rutherford, A. J. & Dieckow, J. (2019). The efficacy of Plantago lanceolata for mitigating nitrous oxide emissions from cattle urine patches. *Science of The Total Environment*, *691*, 430-441.

Vibart, R. E., Vogeler, I., Dodd, M. & Koolaard, J. (2016). Simple versus diverse temperate pastures: aspects of soil–plant–animal interrelationships central to nitrogen leaching losses. *Agronomy Journal, 108*, 2174-2188.

Welten, B. G., Ledgard, S. F., Judge, A. A., Sprosen, M. S., McGowan, A. W. & Dexter, M. M. (2019). Efficacy of different temperate pasture species to reduce nitrogen leaching from cattle urine applied in different seasons: A soil lysimeter study. *Soil Use and Management, 35*(4), 653-663.

Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J. & Clough, T. J. (2018). Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. *Grass and Forage Science, 73*(4), 878-887.

Zaman, M., Saggar, S., Blennerhassett, J. D. & Singh, J. (2009). Effect of urease and nitrification inhibitors on N transformation, gaseous emissions of ammonia and nitrous oxide, pasture yield and N uptake in grazed pasture system. *Soil Biology and Biochemistry, 41*(6), 1270-1280.

2 REVIEW OF THE LITERATURE

Introduction

New Zealand dairy systems are typically based on perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pastures (Charlton & Stewart, 1999) grazed year-round. As a result, the nitrogen (N) inputs of dung and urine are mainly deposited onto the pasture by the grazing animals (Bolan *et al.*, 2004; Haynes & Williams, 1993). Ryegrass-white clover pastures typically have a high N concentration exceeding the nutritional requirements of the grazing animals. Consequently, the excess of N is excreted in the urine. Urine patches from grazed animals are one of the major contributors of N losses due to their small area, providing N loading rates that exceed the uptake capacity of the pasture and soil systems (Haynes & Williams, 1993; Selbie *et al.*, 2015). Therefore, N is prone to be lost from the system as N leached and/or gaseous N emissions (Bolan *et al.* 2004; Di & Cameron, 2002a; Ledgard *et al.*, 2009).

2.1 NITROGEN CYCLE IN GRAZED SYSTEMS

Soils generally contain between 0.1 and 0.6% N in the first 15 cm, representing between 2000 and 12000 kg N ha^{-1} (McLaren & Cameron 1996). Soil N is in the form of organic matter and mineral forms (ammonium-NH_4^+, nitrite-NO_2^- and nitrate-NO_3^-). In grazed pasture systems, the sources of N are biological fixation of atmospheric N, N fertilisers and manures, mineralisation of soil organic N and atmospheric N deposition and N in supply feeds (Bolan *et al.*, 2004; Whitehead, 1995).

Nitrogen is present in soil mainly as part of the soil organic matter, which is not available for plant uptake (McLaren & Cameron, 1996). Nitrogen in the soil undergoes a series of transformations by soil microorganisms that produces different forms of N. These processes are: mineralisation, immobilisation, nitrification, denitrification, adsorption and fixation of NH_4^+, NO_3^- leaching, and ammonia (NH_3) volatilisation (Figure 2.1) (McLaren & Cameron, 1996). The processes of NO_3^- leaching, nitrous oxide (N_2O) and NH_3 emissions are described in section 2.2 as the main N losses from urine patches.

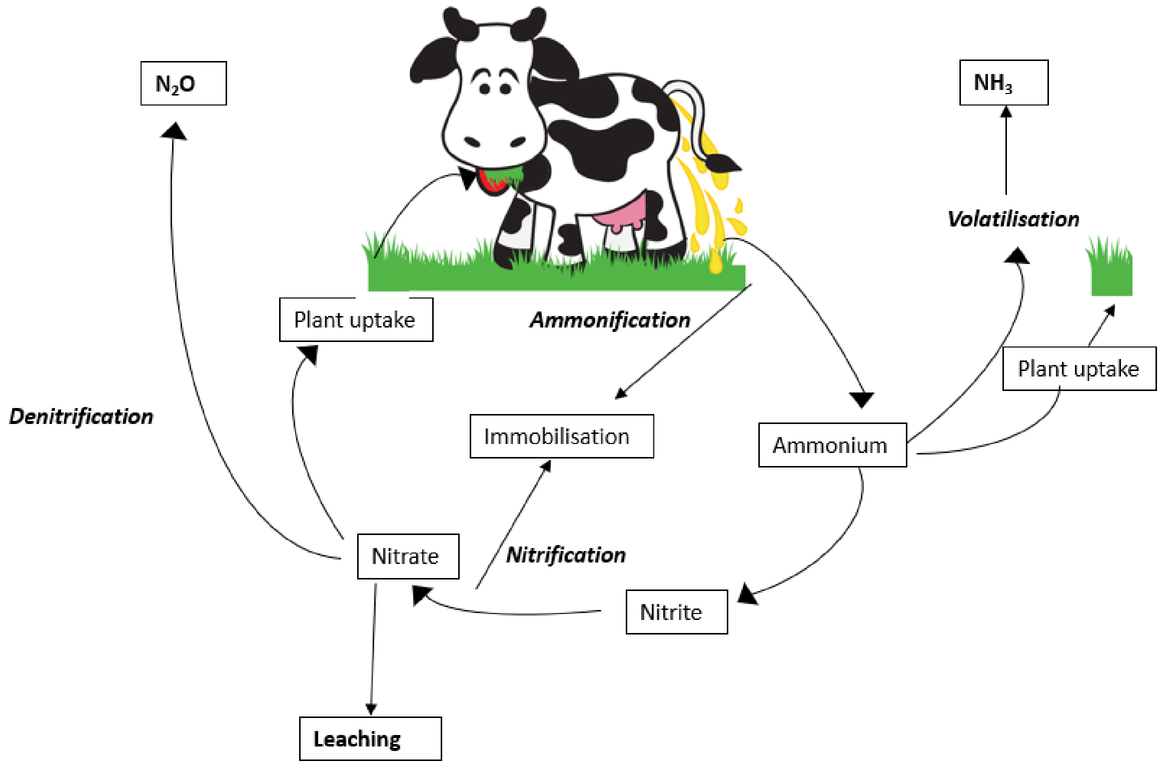


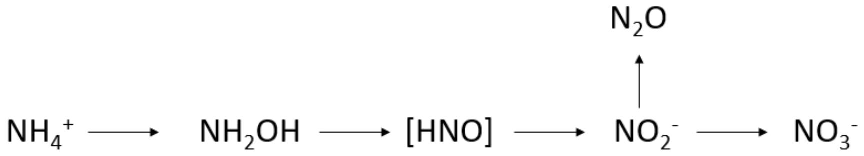
Figure 2.1. Schematic representation of nitrogen cycle.

2.1.1 Mineralisation, immobilisation and nitrification

The process that converts the organic N into inorganic forms available to plants is known as **mineralisation** (McLaren & Cameron, 1996). This process involves the breakdown of complex proteins to amino acids by microbes and these compounds can then be converted into NH_3.

Nitrification is the biological conversion of NH_4^+ to NO_3^- via NO_2^- (McLaren & Cameron, 1996). This process involves two reactions which are carried out by two groups of soil microorganisms. The first step is carried out by the ammonia monooxygenase (AMO) enzyme associated with *Nitrosomonas* and *Nitrosospira* bacteria (Equation 2.1), from NH_4^+ to NO_2^-. During the second step the NO_2^- is oxidised to NO_3^- by *Nitrobacter* (Cameron *et al.*, 2013; Whitehead,1995). The soil microorganisms that carried out this step are autotrophic and obtain their carbon from carbon dioxide (CO_2) and their energy from the oxidation of NH_4^+ to NO_2^- and NO_3^- (McLaren & Cameron, 1996). During this process, hydrogen ions (H^+) are released decreasing the soil pH.

The nitrification process is important in relation to the potential of NO_3^- leaching from the root zone due to it being the only pathway of NO_3^- production (section 2.2.1). During the nitrification process, N_2O can be lost to the air (Davidson & Verchot, 2000). Nitrification is sensitive to changes in soil conditions (McLaren & Cameron, 1996). The soil conditions that most affect the nitrification are soil pH, moisture content, aeration and soil temperature.



$$NH_4^+ \longrightarrow NH_2OH \longrightarrow [HNO] \longrightarrow \overset{\overset{N_2O}{\uparrow}}{NO_2^-} \longrightarrow NO_3^- \tag{2.1}$$

Immobilisation is the reverse process of mineralisation, therefore, the inorganic N is converted into organic N forms (McLaren & Cameron, 1996). Soil microorganisms uptake the mineral N and incorporate it into their tissues. Mineralisation and immobilisation occur simultaneously. The carbon (C) to N ratio (C:N) of the decomposing organic material will determine the net process that occurs. When the N content of the organic material is high, the C:N is low, therefore more N is produced than the N required by microorganisms and net mineralisation takes place (Constantinides & Fownes, 1994). However, when the organic source has low N content, the N released does not meet the N requirements by the microbes, and net immobilisation occurs (Aber & Melillo, 1982). When immobilisation is the dominant process, there is a decrease of the N available not only for plant uptake, but also for losses through leaching and gaseous emissions (McLaren & Cameron, 1996).

2.1.2 Denitrification

Emissions of N_2O, nitric oxide (NO) and dinitrogen (N_2) from soils can occur through the microbial processes of denitrification and nitrification (Arnold, 1954). Biological denitrification occurs under anaerobic conditions such as poorly drained soils (McLaren

& Cameron, 1996). Under this condition, NO_3^- is used as an electron acceptor in place of oxygen by facultative anaerobic bacteria (Saggar *et al.*, 2013).

Equation 2.2 shows the reduction process where NO_3^- is reduced producing NO_2^-, NO, N_2O and N_2 gas. Each step of the reaction requires a specific enzyme, NO_3^- reductase, NO_2^- reductase, NO reductase and N_2O reductase (Saggar *et al.*, 2004).

$$\underset{\text{Nitrate}}{NO_3^-} \longrightarrow \underset{\text{Nitrite}}{NO_2^-} \longrightarrow \underset{\text{Nitric oxide}}{NO} \longrightarrow \underset{\text{Nitrous oxide}}{N_2O} \longrightarrow \underset{\text{Dinitrogen}}{N_2} \qquad (2.2)$$

In grazed pastures, N_2O produced can be lost as gas before the complete reaction takes place. A source of available C is a requirement for the process. The rate of denitrification is also affected by the soil pH with it being very slow in acidic soils, and at low soil temperature (Saggar *et al.*, 2013).

2.1.3 Ammonium adsorption and fixation

Ammonium ions can be adsorbed by the cation exchange site to the clay surface and organic matter in soils due to their positive charges. Ammonium can also be fixed in the interlayers of 2:1 clay minerals in a non-exchangeable fixed form (McLaren & Cameron, 1996). Therefore, NH_4^+ is less mobile and less prone to leaching than NO_3^-.

2.2 THE URINE PATCH

Urine patches are small areas where urine is deposited by the grazing animals. These patches are often visible in a paddock due to the darker green pasture compared with the surrounding area (Moir *et al.*, 2011). The area covered by the urine patch of cows is widely variable, averaging 0.24 m^2 per patch (Selbie *et al.*, 2015) and covering between 10 to 30% of the total area grazed by the animals per annum (Moir *et al*,. 2011).

The urination volumes can be highly variable and range from 0.30 to 7.83 L/event, (Betteridge *et al.*, 2013) with an average of 2.1 L/event (Selbie *et al.*, 2015). The frequency of urination is on average 8-12 events per day (Haynes & Williams, 1993).

Urine N concentration (UNc) can range between 2 to 20 g N L^{-1}, with an average of 6.9 g N L^{-1} in dairy cattle (Dijkstra *et al.*, 2013; Selbie *et al.*, 2015).

The main factors that affect urinary N concentration are the N content in feed and the water intake by grazing animals (Dijkstra *et al.*, 2013). Generally, there is a linear relationship between N intake and N output in urine and an increase in N intake results in higher N losses in the urine (Olmos Colmenero & Broderick, 2006; Dijkstra *et al.*, 2013). An increase in water intake can reduce UNc through a dilution effect (Ledgard *et al.*, 2015), therefore, an increase in N intake may not always result in a high UNc.

Excess of N arises when animals are fed diets with crude protein (CP; N x 6.25) contents above 18-20% (Pacheco & Waghorn, 2008). In pastoral dairy systems, between 60-90% of N intake by grazing animals is excreted into the soil, of which 70% is urine and between 50-90% of the N in urine is in the form of urea (Bolan *et al.*, 2004; Cameron *et al.*, 2013; Haynes & Williams, 1993; Whitehead, 1995). Nitrogen concentrations in urine are much higher than plant N requirement, therefore, the excess of N which remains in soil is prone to be lost as NO_3^- leaching during rainfall events; and N gases such as N_2O and NH_3, which will have a negative impact on the environment (Carter, 2007; Haynes & Williams, 1993; Sherlock & Goh, 1984; Stark & Richards, 2008).

Urine patches are ‘hot-spots’ where readily available C and N are applied locally and in high concentration, creating the conditions for N loss from the system (Bol *et al.*, 2004; Saggar *et al.*, 2004, 2013). Of total urinary-N losses, NO_3^- leaching, NH_3 volatilisation and N_2O emissions account for an average of 18, 13 and 2%, respectively, and plant uptake and immobilisation account for 41 and 26%, respectively (Selbie *et al.*, 2015).

2.2.1 Nitrate leaching

In agricultural soils, urine patches are the main source of NO_3^- losses (Haynes & Williams, 1993). Several studies have been conducted to evaluate NO_3^- leaching from dairy pasture systems with different soil types (Christensen *et al.*, 2018; Di & Cameron, 2002a; Houlbrooke *et al.*, 2003; Pakrou & Dillon, 2004). The amount of NO_3^- leached is determined by the amount of NO_3^- accumulated into the soil, the N uptake by plants, N transformations into the soil and the drainage volume (Di & Cameron, 2002b). Due to nitrate’s negative charge, it is not retained by cation exchange sites in soils, being leached when water drains through the soil (McLaren & Cameron, 1996). Nitrate that is not taken up by plants can be lost from the system and enter into ground water and streams where in high concentration it is a threat to the environment and human health (Di & Cameron, 2002b; Stark & Richards, 2008; Whitehead, 1995). Losses of NO_3^- leaching from urine patches can range between 12% to 58% of N applied in urine (Di & Cameron, 2002a; Malcolm *et al.*, 2014; Shepherd *et al.*, 2011; Silva *et al.*, 1999).

Nitrate leaching losses mainly occur during autumn and winter, when soil moisture is higher than field capacity (Christensen *et al.*, 2018). During this time of the year, there is an excess of water, evapotranspiration rates are low and plant uptake of NO_3^- is low, therefore, NO_3^- in soil solution is prone to be leached (Buckthought *et al.*, 2015; Di & Cameron, 2002b). In a field experiment in Tokomaru soil with a mole and pipe drainage system, it was reported that after a grazing event NO_3^- and total N in the drainage water were 5 times higher than the levels before grazing (Sharpley & Syers, 1979). In the same soil type as Sharpley & Syers (1979), Christensen *et al.* (2018) reported high levels of NO_3^- at the beginning of the drainage season during three consecutive years 12.9, 9.0 and 19.1 mg L^{-1}, which then decreased to values lower than 2 mg L^{-1} after 200 mm of cumulative drainage. High levels of NO_3^- at the beginning of winter, are due to the N inputs from fertilisers, urine, fixation and mineralization, are higher than plant N uptake and so that N is accumulated in soil profile where it is prone to be leached (Christensen *et al.*, 2018).

The ‘critical period’ for urine deposition in the soil has been defined as the period which strongly influenced the NO_3^- leaching losses to the drainage water. Studies have determined that the critical period is from late summer to early winter, due to the accumulation of the N into the soils (Christensen, 2013; Shepherd *et al.*, 2011, 2017). Results suggest that when urine is deposited in late summer it is more susceptible to be leached in winter (Shepherd *et al.*, 2011). Shepherd *et al.*, (2011) observed low pasture growth when urine was applied in February due to a dry summer which affected the N uptake by the pasture. In a lysimeter experiment, where urine was deposited in spring, summer and autumn, the amount of NO_3^- leached increased the later the urine was deposited regardless of the soil type (Decau *et al.*, 2003). The authors suggested that the earlier the urine is deposited during the grazing season, the greater the chance the N can be utilized by the pasture.

2.2.2 Ammonia volatilisation

The urea-N in urine is hydrolysed by the urease enzyme to ammonium carbonate [$(NH_4)_2CO_3$], which is unstable and is dissociated into NH_4^+-N and carbonate (CO_3^{2-}) (Equation 2.3) (Bolan *et al.*, 2004; Sherlock & Goh, 1984). Then, CO_3^{2-} ions react with water to form bicarbonate (HCO_3^-) and hydroxyl ions (OH^-) which increase the soil pH to values greater than 7.2 (Saggar *et al.*, 2004). Ammonium ions dissociate into NH_3 gas which is subject to volatilisation losses (Bolan *et al.*, 2004).

$$(NH_2)_2CO + 2\,H_2O \xrightarrow[\text{urease}]{} (NH_4)_2CO_3 \longrightarrow NH_4^+ + CO_3^{2-}$$

$$CO_3^{2-} + H_2O \longleftrightarrow HCO_3^- + OH^-$$

$$NH_4^+ + OH^- \longleftrightarrow NH_{3(g)} + H_2O \qquad (2.3)$$

The conversion of NH_4^+-N to NH_3 gas controls the volatilisation rate, which occurs under alkaline conditions (Bolan *et al.*, 2004; Sherlock & Goh, 1984). The difference in partial pressure of NH_3 between soil and the atmosphere is one of the factors that drives the NH_3 volatilisation process (Bolan *et al.*, 2004; Saggar *et al.*, 2004).

Urease enzyme is responsible for the hydrolysis of urea into NH_4^+-N which occurs within one to two days of application of urea/urine (Zaman *et al.*, 2008, 2009, 2013). Several environmental and soil factors at the time of deposition of the N source affect the rate of urea hydrolysis. The environmental factors that most affect NH_3 volatilisation are wind speed, rainfall and temperature (Whitehead & Raistrick, 1991). Soil temperature, pH, cation exchange capacity (CEC), buffering capacity and water content are the soil factors which affect the rate of NH_3 emissions (Bolan *et al.*, 2004; Saggar *et al.*, 2004; Whitehead & Raistrick, 1993). Ammonia losses from urine patches can range from 7 to 14% of the N applied in the urine (Rodriguez *et al.*, 2019; Zaman & Blennerhassett, 2010; Zaman & Nguyen, 2012).

2.2.3 Nitrous oxide emissions

In livestock systems, N_2O is produced from soils mainly by the microbial processes of nitrification and denitrification (Butterbach-Bahl *et al.*, 2013; de Klein *et al.*, 2010; Saggar *et al.*, 2004; Wrage *et al.*, 2001).

Urine deposition in soils increases N_2O emissions and the main factors that affect the N_2O production from soil are: water-filled pore space (WFPS), soil pH and NO_3^- concentration, and available soil C (Butterbach-Bahl *et al*., 2013; de Klein & Eckard, 2008; Haynes & Williams, 1993; van Groenigen, *et al*., 2005; Whitehead, 1995). In grazing systems, high NO_3^- concentration in soils and low oxygen (O_2) availability are the key factors affecting N_2O production. Trampling and treading by animals can enhance the rate of N_2O production due to compaction in soils especially under wet conditions (Oenema *et al.*, 1997).

In New Zealand, to calculate the annual N_2O emissions from urine, the Intergovernmental Panel on Climate Change (IPPC) inventory calculation method has been adopted, and the current emission factor is 1% of the urine-N applied (Ministry for the Environment, 2018), which represents the N_2O-N emitted as % of the N applied (EF_3). Several field experiments have been conducted to provide data to calculate the EF_3 in different soils and environmental conditions. The N_2O emission factor for animal urine in agricultural soils in New Zealand ranges from 0.1 to 4% of the N applied (de Klein *et al.*, 2001, 2003). The variability between the EF_3 from urine patches is mainly attributed to the conditions where urine is deposited, soil drainage class and environmental conditions, as well as the length of measurement period (de Klein *et al.*, 2003). For example, de Klein *et al.* (2014) determined that the EF_3 when synthetic urine was deposited in free-draining soils ranged between 0.10 and 0.34%, and 0.21 to 0.9% in a poorly drained soil.

2.3 ENVIRONMENTAL IMPACTS OF NITROGEN LOSSES FROM URINE PATCHES

In New Zealand, the intensification of livestock systems has a major environmental impact, including increased NO_3^- leaching, and N_2O and NH_3 emissions (Bolan *et al*., 2004; Di & Cameron, 2002b, 2016; Haynes &Williams, 1993; Selbie *et al*., 2015). Nitrate leaching mainly affects water quality in groundwater, rivers, lakes and streams quality (Stark & Richards, 2008). High concentrations of NO_3^- in these aquatic systems can cause eutrophication and algal blooms (Leip *et al*., 2015). Nitrate-N concentration higher than 11.3 mg L^{-1} in drinking water is also considered a risk to human health (World Health Organisation, 2011). The issues associated with NO_3^- concentration greater than the World Health Organisation threshold in drinking water are methaemoglobinaemia (blue baby syndrome) in babies and an increasing risk of stomach cancer (World Health Organisation, 2011). Therefore, it is extremely important to keep NO_3^- concentrations below this threshold to protect human health and to reduce the environmental footprint of agriculture without negative impact on farm production and profits.

In terms of N losses in grazed systems, urine patches are also the major source of these emissions. Due to the high urea-N concentration in urine, high losses of NH_3 volatilisation occur over the first 48 hours after urine deposition. These N losses not only produce eutrophication of waterbodies, but also act as a second source of N_2O, which is a greenhouse gas (GHG) (Saggar *et al*., 2005). Nitrous oxide is a potent GHG which contributes to global warming and has also been described as an ozone-depleter (Ravishankara *et al*., 2009). In New Zealand, agriculture accounts for 49% of the GHG emissions and 22% of these emissions are N_2O (Ministry for the Environment, 2018).

Although agriculture is the largest contributing sector to New Zealand’s greenhouse gas emissions, on a global scale, New Zealand’s total emissions are small (Mazzetto *et al*., 2021). New Zealand is the most efficient producer of low emissions milk at 0.77 kg CO_{2e} per kg of fat and protein corrected milk (FPCM). This represents 48% less than the average (1.47 kg CO_{2e} per kg FPCM) of the countries included in the study (Mazzetto *et al*., 2021). A combination of improving grassland management and feeding practices with improved animal genetics and management mean that farms are using resources more efficiently to increase the farm outputs.

Strategies have been recommended as mitigation tools to reduce N losses from urine patches. In the next section these strategies will be discussed with particular emphasis on the inclusion of plantain in the diet of animals due to it being the main focus of this thesis.

2.4 STRATEGIES TO REDUCE NITROGEN LOSSES FROM URINE PATCHES

Several options have been investigated to reduce N losses from urine patches whilst maintaining or increasing farm production (Di and Cameron, 2001; Martin, 2018). Some strategies aim to reduce N load in urine patches and hence less N will be lost from the system. Other strategies aim to reduce urine patches deposited into the soil or to modify the N cycle in soils. Controlling the time that cows spend grazing in the paddocks during autumn/winter will reduce the urine deposition in the paddock. Another approach is the use of inhibitors to modify the N cycle in soils, reducing the rate of nitrification.

2.4.1 Interventions in feed supply (High energy low crude protein)

An increase in the N intake by animals will increase the amount of N excreted in urine (Castillo *et al*., 2000; Kebreab *et al*., 2001). Therefore, a potential method to reduce N in the urine patch is to manipulate the animals' diet to reduce the N intake. Diet needs to be carefully balanced to not affect the feed intake and the milk production of animals.

The CP requirement for lactating cows ranges from 14 to 18 % of dry matter (DM) intake (Pacheco & Waghorn, 2008). In New Zealand, the diet of dairy cows consists mainly of high-quality ryegrass-white clover (Dillon *et al.*, 2005) with CP content up to 30% of DM (Moller *et al*., 1996). This excess of protein is transformed to urea in the animals and mainly excreted as urea-N in the urine (Whitehead, 1995). Reducing the CP content in the diet of animals is a potential strategy to decrease the N loss to the water and to the environment. Reductions in UNc and urine N excretion have been observed when the CP content of cows' diet was reduced (Kyamanywa *et al.*, 2020; Misselbrook *et al*., 2005).

Reducing UNc through a change in the diet of dairy cows is clearly an alternative to the use of common ryegrass-white clover pastures without compromising milk production. However, it can involve an extra cost because it requires bringing extra supplements onto the farm. Alternatively, this approach requires a complex strategy of growing low CP annual crops to balance against daily ryegrass-white clover intake, which brings an increase in risk of crop failure, workload and management complexity to a farm system.

2.4.2 Duration controlled grazing

Duration-controlled (DC) grazing aims to reduce the amount of N leached by reducing the urine and dung patches deposited by the cows on grazing pastures compared to conventional grazing (Christensen *et al.*, 2018; de Klein & Ledgard, 2001). After cows graze to the required residual, cows are moved to stand-off facilities where they receive supplements if needed (Christensen, 2013; de Klein, 2001). The effluent collected during the time cows are on the stand-off facilities is stored and then later returned to the paddocks. The effluent is evenly spread through the paddock thereby decreasing the N load, and plants can utilise the N contained it.

Duration-controlled grazing significantly reduced N leached and treading damage to the soil during wet periods (Christensen *et al.*, 2018; de Klein & Ledgard, 2001). . Christensen *et al.* (2018) reported a 52% reduction in N leaching using year-round DC grazing system compared to a conventional grazing system. Restricted autumn/winter grazing reduced NO_3^- leaching by 40% (de Klein *et al.*, 2006). If the DC grazing is carried out during late summer and autumn/winter, a significant decrease in NO_3^- leaching could be achieved during a short period of time which has been known as ‘the critical period’ (Christensen *et al.*, 2018).

During DC grazing, the time cows spend in stand-off facilities could be used as a feeding strategy to supplement cows with silage with low CP content, reducing the ingested N in the diet of cows. The main disadvantages of this strategy are the extra cost of building of the facilities, the storage and spreading of the effluent, making the silage to feed the cows or buying the feed, and the NH_3 volatilisation from the effluent stored.

Other strategies to reduce the UNc used are the management of N fertiliser and the rotation length/ regrowth period which are reviewed by Martin (2018).

2.4.3 Nitrification inhibitors

Nitrification inhibitors (NI) have been developed as a mitigation strategy to reduce N losses from urine patches and N fertilisers, by modifying the N cycle in the soil (de Klein & Eckard, 2008; Di & Cameron, 2002c; Stark & Richards, 2008). The NI are chemical compounds that slow the conversion of NH_4^+ to NO_2^-, inhibiting the activity of the ammonia- oxidizing bacteria (AOB) in soils (Equation 2.5) (Di & Cameron, 2002c, 2016).

The nitrification rate is slowed and N is kept in the form of NH_4^+ which can be absorbed by the soil exchange surfaces and/or taken upby plants (Di & Cameron, 2002c; Zaman *et al.*, 2009; Zaman & Blennerhassett, 2010).

$$NH_4^+ \xrightarrow[\text{NI}]{\times} NH_2OH \longrightarrow HNO \longrightarrow NO_2^- \longrightarrow NO_3^- \qquad (2.5)$$

Dicyandiamide (DCD) is a NI that slows down the conversion of NH_4^+ to NO_3^-, keeping N in soils in a less leachable form. However, in 2013, DCD was withdrawn from use in New Zealand due to traces of it being found in milk products (Ministry for Primary Industries, 2013). Nowadays, there is no commercial available NI.

The use of NI implies an extra cost for farmers. The NI are usually applied with fertilisers, however, it is important to know the fate of the inhibitors after application due to their potential to be accumulated in the pastures and subsequently in animal products such as milk and meat.

2.4.4 New interventions involving plantain

A number of studies have evaluated diverse swards containing plantain and reported a reduction in UNc in cows fed plantain swards compared to ryegrass-white clover swards (Al-Marashdeh *et al.*, 2019; Box *et al.*, 2017; Cheng *et al.*, 2017b; Dodd *et al.*, 2018; Edwards *et al.*, 2015; Judson & Edwards, 2016; Minnée *et al.*, 2017, 2020; Totty *et al.*, 2013; Woodward *et al.*, 2012). Including plantain in the cows' diet could be a natural mitigation strategy to reduce the N losses from dairy farms to the environment in a more cost-effective manner. The lower UNc of cows grazing pastures containing plantain creates an opportunity to reduce the urinary load of grazing cows (Totty *et al*., 2013; Box *et al*., 2018; Bryant *et al*., 2016).

Evidence shows that plantain can alter the soil N cycling due to containing secondary metabolites which can inhibit the nitrification process in soils (Dietz *et al.*, 2013), acting as biological nitrification inhibition (BNI) (Subbarao *et al.*, 2006). These secondary metabolites in plantain can be divided in two categories, iridoid glucosides (IG) and phenylethanoid glucosides (Stewart, 1996; Tamura & Nishibe, 2002).

The main compounds in the IG category are catalpol and aucubin, whereas acteoside is a phenylethanoid glucoside (Tamura & Nishibe, 2002). Secondary metabolites are organic compounds produced by plants which are not essential for the main functions of the plants, usually having antimicrobial activities (Bourgaud *et al.*, 2001). The concentration of these secondary metabolites is affected by temperatures, leaf stages and cultivars of plantain (Box *et al*., 2018; Tamura & Nishibe, 2002). Catalpol is present in the cultivar 'Grassland Lancelot', however, it is absent in the cultivar 'Ceres Tonic' (Al-Mamun *et al.*, 2008; Tamura & Nishibe, 2002). Acteoside and aucubin are present in the different cultivars of plantain. Concentration of acteoside in leaves is variable and ranges from 3.2 mg g^{-1} DM to 41 mg g^{-1} DM in the cultivar 'Ceres Tonic' (Al-Mamun *et al*., 2008; Navarrete *et al*., 2016; Tamura & Nishibe, 2002). Aucubin concentration is lower than acteoside and ranges between 7.9 to 13.1 mg g^{-1} DM.

2.4.4.1 Urinary nitrogen effects

Reducing UNc can lead to a reduction in N losses at a farm scale. The urine N load into the soil is affected by the UNc (g N L^{-1} urine) and urine volume (L of urine per urination event) (Ledgard *et al.*, 2009). A change in the cows' diet can influence the N content and concentration in urine which will affect the amount of N available for soil nitrification and denitrification processes.

Studies on diverse mixed swards containing at least 20% plantain have been shown to reduce the N concentration in spot-sampled urine from dairy cows (Al-Marashdeh *et al.*, 2019; Box *et al*., 2017; Cheng *et al.*, 2017b; Dodd *et al.*, 2018; Edwards *et al.*, 2015; Judson & Edwards 2016; Minnée *et al.*, 2017; Totty *et al.*, 2013; Woodward *et al.*, 2012). Some studies also suggested that the total N excretion from cows (g N/day/cow) could be reduced (Box *et al.*, 2017; Navarrete *et al.*, 2020; Totty *et al.*, 2013). Table 2.1 summarises studies (field and metabolism stalls) conducted in New Zealand, which reported the effect of including plantain in the cows' diet or heifers'diet and the effect on UNc and milk yield. Figure 2.2 shows the relationship between the % of plantain offered to the cows and the effect on UNc. When the % of plantain increased, the UNc decreased, however, the relationship is weak.

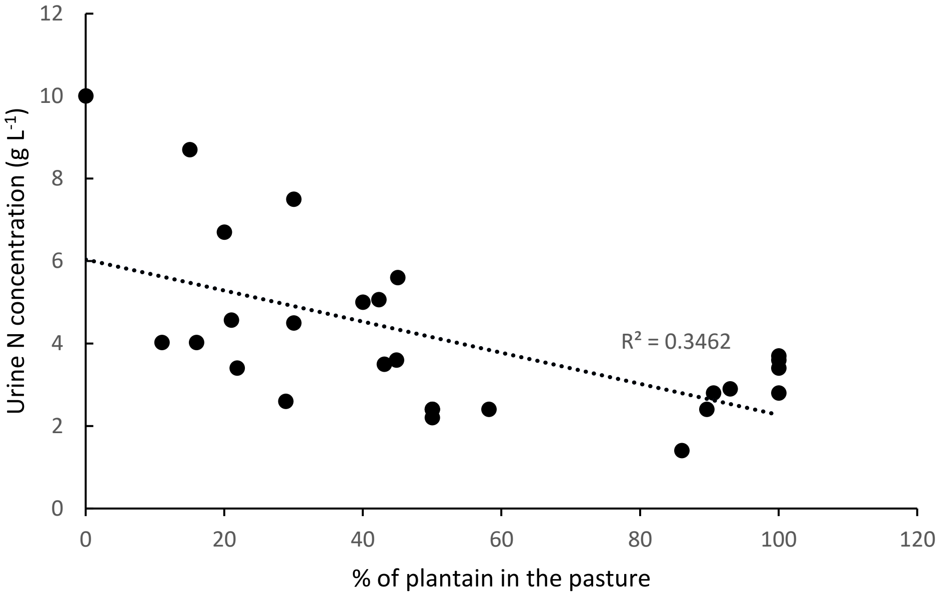


Figure 2.2 Effect of percentage of plantain in the pasture grazed by dairy cows on the UN concentration (g L^{-1}). The data used for the graphs is from Table 2.1.

In a confinement study, reductions of up to 50% in UNc were observed when cows were fed diverse swards including plantain compared to ryegrass-white clover swards. The reduction in UNc could be due to a reduction in the DM content and N concentration in the DM of the pasture including plantain (Woodward *et al.*, 2012). Minnée *et al.* (2020) also reported reduction in the UNc when cows were fed diets including 30 and 45% of plantain and it was attributed to a dilution effect and alteration in N partition. This result was obtained after full collection of urine from cows. The reasons suggested for the reduction in UNc and N excretion in cows grazing plantain are: a reduction in the N intake by cows fed swards containing plantain; a dilution effect due to an increase in frequency of urination ; more N eaten partitioned to the faeces; or an inhibitor effect of the secondary metabolites produced by plantain which can reduce the UNc.

The N intake is the main driver of the N lost in cows' urine (Kebreab *et al*., 2001). Studies where plantain was incorporated into cows' diet did not show a linear relationship between the N intake and UNc (Box *et al*., 2017; Minnée *et al*., 2017, 2020; Totty *et al*., 2013). In a stall study, Minnée *et al*. (2020) observed a reduction in UNc of 19 and 41% in cows fed 30 and 45% plantain, respectively, compared to ryegrass-white clover swards, and the N intake across the diets was similar. Reductions in UNc were also observed in a grazing study were cows were fed 50 or 100% plantain (Box *et al*., 2017). The reductions in UNc could be explained as a dilution effect (Mangwe *et al*., 2019; Minnée *et al*., 2020; O'Connell *et al*. 2016). Mangwe *et al*. (2019) observed lower UNc from cows fed plantain compared to ryegrass-white clover swards. This was attributed to an increase in the number of urination events by cows grazing plantain compared to ryegrass-white clover swards, in agreement to the results found by Minnée *et al*. (2020). The inclusion of plantain to the diet of sheep also increased the volume of urine by 0.5 L compared to sheep fed ryegrass-white clover during the experimental period (O'Connell *et al*., 2016).

When plantain was included in the diet of dairy cows, some researchers observed that more N was partitioned to the dung, reducing the UNc (Dodd *et al*., 2018; Minnée *et al*., 2020). Incorporating plantain into the cows' diet in spring, increased the faecal N concentration, which was 18% greater from cows grazing swards containing plantain (Dodd *et al*., 2018). Similarly, Minnée *et al*. (2020) reported an increase in N concentrations in the faeces of cows fed 30 and 45% plantain compared to cows fed 15% plantain and ryegrass-white clover swards. There are other studies that did not report any differences in faecal N concentrations (Box *et al*., 2017; Judson & Edwards, 2016; Minnée *et al*., 2017; Totty *et al*., 2013; Woodward *et al*., 2012). The effect of plantain on the N partitioned to dung could lead to lower N losses from urine patches.

There are some plant characteristics that increase the N partitioning to dung (Bryant *et al*., 2019; Minnée *et al*., 2019). Minnée *et al*. (2019) reported that the soluble N concentration and non-structural carbohydrates affect the N partitioning in the cows. Reductions in the soluble and degradable N could reduce rumen ammonia (NH_3) concentrations of cows fed plantain compared with cows fed ryegrass-white clover. This difference could explain the increase in N partitioning to faeces where it is less prone to be leached as NO_3^- or N_2O. The non-structural carbohydrates (NSC) are a source of energy for rumen microbes. Minnée *et al*. (2019) observed that plantain has a greater concentration than ryegrass-white clover pastures. The ratio NSC to N in the animal diet has an effect on the N partitioning in cows, reducing the proportion of dietary N eaten partitioned to urine.In an *in vitro* incubation, Navarrete *et al.* (2016) showed that aucubin decreased the production of NH_3, suggesting aucubin can be degraded to its active form aglycone aucubigenin in the rumen (Berenbaum & Rosenthal, 1992) with the potential to reduce UNc. Navarrete *et al*. (2016) found that the concentration of both aucubin and acteoside increased from September to April. Tamura and Nishibe (2002) observed that high air temperatures increased the concentration of IG compounds and lower temperatures enhanced the concentration of acteoside.

2.4.4.2 Dry matter production of plantain

Herbage production of plantain ranges between 7.5-12 t DM ha^{-1} (Navarrete, 2015; Powell *et al.*, 2007; Rumball *et al*., 1997; Stewart, 1996). The greatest accumulation of DM production is during late spring and summer due to plantain's drought tolerance (Stewart, 1996).

The DM production from a diverse sward containing plantain or from a pure plantain sward can be as high productive as a ryegrass-white clover sward. In a field experiment, where plantain was included in a mix sward with white and red clovers (*Trifolium pratense*) and ryegrass, plantain provided a significant advantage of 6 t DM ha^{-1} and 1.2 t DM ha^{-1} in year 1 and 3, respectively, compared to ryegrass-white clover swards (Moorhead & Piggot, 2009). Plantain-based swards yielded 1.8 and 0.9 t DM ha^{-1} more than ryegrass swards, in summer and autumn, respectively (Moorhead & Piggot, 2009). Similarly, in a field experiment in Canterbury, the annual DM production during two years, was 1.62 t DM ha^{-1} greater in a diverse sward containing plantain and chicory than in ryegrass-white clover swards (Nobilly *et al.*, 2013). The authors attributed the differences to the greater growth of the diverse swards during summer due to a high percentage of the herbs in the swards during that period. Both plantain and chicory accumulate the greatest herbage mass during summer due to drought tolerance (Lee *et al*., 2015; Powell *et al*., 2007). The major factor affecting the persistence of plantain in a pasture are plant density and loss of plants over time (Navarrete, 2015; Tozer *et al*., 2011), however, there is still limited information on plantain persistence and grazing management on dairy farms.

2.4.4.3 Milk yield

The effect of including plantain in the diet of dairy cows on milk production has been studied in diverse and in pure plantain swards with variable results (Table 2.1).

Plantain has the potential to increase milk production in late spring and autumn. Mangwe *et al.* (2020) compared milk solids production of cows fed on plantain and ryegrass-white clover swards. Despite the DM intake being similar for both treatments, the milk solids production was greater in cows grazing plantain than ryegrass-white clover swards. Woodward *et al.* (2012) reported that milk solids production was higher in diverse swards containing plantain than ryegrass-white clover, with lower DM intake in the diverse swards compared to ryegrass-white clover. Box *et al.* (2017) also reported an increase in milk solids production in autumn from cows fed plantain compared to ryegrass-white clover. In the experiment carried out by Box *et al*. (2017), the DM intake increased approximately 20% in cows grazing plantain in autumn compared to ryegrass-white clover, but no difference in spring was observed. Minnée *et al*. (2020) reported that milk and MS yield were greater when plantain comprised more than 30% of the diet of the cows. However, Totty *et al.* (2013) did not find any difference in milk solids production in autumn when cows grazed either ryegrass-white clover, high-sugar ryegrass-white clover or diverse swards containing plantain. Similar results were observed by Edwards *et al.* (2015), and Dodd *et al.* (2018). From these experiments, overall milk solids production from cows, grazing diverse swards containing plantain or pure plantain swards, was similar or greater than milk solids production from cows grazing ryegrass-based pastures.

The studies reported above were short-term experiments containing plantain, showing that including plantain in cows’ diet can improve milk production. However, there is no long-term experiment, over a whole grazing season, that studies the effect of plantain on animal production compared with a standard ryegrass-white clover sward.

Table 2.1. A summary of studies conducted in New Zealand that determine the effect of including plantain in animals' diet on UN concentration.

Treatments	**Season**	**Milk solids yield**	**UN concentration (g L^{-1})**	**Faecal N**	**N intake (g N/heifer/day)**	**Apparent DMI (kg/cow/day)**	**Authors**
-RG/WC	Autumn	1.50^{a}	5.4^{a}	3.43(%)	619	13.5	Box *et al.* (2017)
-PL (89.6%)	Autumn	1.67^{b}	2.4^{c}	3.45	594	15.9	
-50% RG/WC + 50% PL (89.7%)	Autumn	1.60^{ab}	3.6^{b}	3.33	553	14.9	
- RG/WC	Spring	2.42	4.7^{a}	3.97^{a}	652	18.5	
-PL (67.8%)	Spring	2.39	2.2^{c}	3.60^{b}	669	20.7	
-50% RG/WC + 50% PL (66.7%)	Spring	2.43	3.4^{b}	3.85^{a}	629	18.5	
- RG/WC	Early autumn	1.54	0.51a (%)	3.35 (%)	604	16.5	Bryant *et al.* (2018)
-Mix pastures (RG/WC, chicory, PL (16%), Lucerne)		1.64	0.43b	3.51	637	15.2	
- RG/WC	Autumn		2.1(g/kg)		187	6.3	Cheng *et al.* (2017b) Field exp.
- RG/WC +PL50%	Autumn		2.4		148	5.4	
-PL	Autumn		2.8		141	5.3	
- RG/WC	Spring		4.8		348	10	
- RG/WC +PL50%	Spring		3.5		294	9.5	
-PL	Spring		2.9		225	7.2	
- RG/WC			3.5 (g/kg)		120	4.9	Cheng *et al.* (2017a) Metabolism stall
-PL			1.4		125	5.2	
-RG+ LU (60:40)	Summer	1.09	7.0^{a}	25(g/kg DM)	642	15.0	Dodd *et al.* (2018)
-RG/LU+PL(60:20:20) (51%PL)	Summer	1.02	3.8b	25	487	15.0	
-Tall fescue (TF)+ LU (60:40)	Summer	1.09	6.3a	24	629	15.3	
-TF LU+ PL (60:20:20) (39% PL)	Summer	1.20	4.5^{b}	25	497	14.9	
-RG+ LU (60:40)	Spring	1.49	6.2^{a}	32	494	14.9	
-RGL+ PL (60:20:20) (51%PL)	Spring	1.44	4.9^{b}	35	497	16.5	
-TF+ LU (60:40)	Spring	1.46	6.1^{a}	30	599	16.7	

-TFLU+ PL (60:20:20) (37%PL)	Spring	1.51	4.8^{b}	33	610	16.5	
- RG/WC -Diverse (RGW, PL, chicory and LU) 15.1% herbs	Summer	2.09 (kg/cow/d) 1.94	6.1^{a}(g/L) 4.9^{b}	3.6^{a}(g/kg DM) 3.3^{b}			Edwards *et al.* (2015)
-Kale+ RG (baleage) -Kale+ PL (baleage)		0.58 0.36	0.58^{a}(%) 0.36^{b}	2.1 % 2.3	172 179		Judson and Edwards (2016)
- RG/WC - RG/WC PL20 - RG/WC PL40		0.93 0.98 0.98	0.70(%) 0.67 0.50			15.2^{a} 15.0^{a} 13.6^{b}	Minnée *et al.* (2017) Indoor exp.
- RG/WC -High sugar RGW (HS) -Diverse pasture mix (chicory, PL, lotus, HS)-18.4% PL		1.55 (kg/d) 1.49 1.47	0.57^{a} (%) 0.58^{a} 0.34^{b}	3.6^{a} (%) 3.4^{b} 3.5^{a}	589.7 (g/d) 609.9 550.7	14.0 14.5 14.5	Totty *et al.* (2013)
- RG/WC -Mixed pastures (RG/WC, PL, chicory, LU)			0.62^{a} (%) 0.26^{b}	2.84 2.83	466^{a} 250^{b}	15.8^{a} 14.6^{b}	Woodward *et al.* (2012) metabolism stall

- RG/WC: Ryegrass-white clover; PL: plantain; LU: Lucerne; TF: tall fescue

2.4.4.4 Effect of plantain sward on soils

Plantain can be used as a potential strategy to reduce N_2O emissions and NO_3^- leaching from dairy systems. Not only by reducing UNc in cows fed plantain, but also as a BNI in the soils. Research in New Zealand, laboratory and field experiments, has focused on the potential of plantain to reduce N losses from urine patches (Carlton *et al.*, 2018; Di *et al.*, 2016; Gardiner *et al.*, 2017, 2019a, 2019b; Judson *et al.*, 2019; Luo *et al.*, 2018; Malcolm *et al.*, 2014; Podolyan *et al.*, 2019; Simon *et al.*, 2019; Welten *et al.*, 2019; Woods *et al.*, 2018).

Aucubin, one of the IG produced by plantain, has exhibited BNI properties (Dietz *et al.*, 2013; Gardiner *et al.*, 2017, 2019a, 2019b). Plantain leaf extract (PLE) added to soils resulted in lower NO_3^- concentration and higher NH_4^+ accumulation compared with soils without the PLE, indicating inhibition of nitrification (Dietz *et al.*, 2013). This inhibition process is carried out by the aglycone of aucubin, aucubigenin (Bartholomaeus & Ahokas, 1995; Gardiner *et al.*, 2016). Aucubigenin can inhibit the enzyme cytochrome P-450, which is a hemeprotein complex related to the ability to block the AMO responsible for ammonia oxidation in soils (Bartholomaeus & Ahokas, 1995; Davini *et al.*, 1986).

It has been hypothesised that plantain could affect N_2O emissions due to the exudation of BNI by the roots (de Klein *et al.*, 2019; Gardiner *et al.*, 2016; Luo *et al.*, 2018; Simon *et al.*, 2019). Luo *et al.* (2018) reported that N_2O reductions ranged from 39 to 74% when urine from cows (fed ryegrass-white clover) was applied to pure plantain swards compared to ryegrass-white clover swards. The effect of the plantain sward when the same urine was applied to swards with different proportions of plantain, was a linear reduction in cumulative N_2O emissions with increasing proportion of plantain (Simon *et al.*, 2019). Although there were significant reductions in N_2O emissions in those experiments, soil mineral N results did not show any inhibition in nitrification. Contrary to these results, in a free-draining soil where the same urine loading rate was applied to swards with 0, 30 and 100% of plantain, no reduction in N_2O emissions or nitrification was reported (Podolyan *et al.*, 2019).

As described in section 2.4.4.1, the inclusion of plantain in the cows' diet reduces the UNc which could also have an effect by reducing N_2O and NO_3^- losses from soils. Di *et al.* (2016) found a 46% reduction in N_2O emission when urine with a N load rate of 500 kg N ha^{-1} (simulating urine from diverse swards including plantain) was applied to the soil compared to a ryegrass-white clover urine with a N load rate of 700 kg N ha^{-1}. Podolyan *et al.* (2019) also reported a 30% reduction in N_2O emissions from the urine loading rate of 450 kg N ha^{-1} compared to a loading rate of 700 kg N ha^{-1}. In those experiments, the reduction in N_2O emissions was due to a decrease in the UNc of cows fed plantain compared to cows fed ryegrass-white clover swards. Judson *et al*. (2019) reported that urine from cows fed plantain inhibited the nitrification rates over the first month after application compared with urine from cows fed ryegrass-white clover swards. The experiment carried out by Judson *et al*. (2019) shows that not only do plantain swards have an effect on the nitrification process, but also urine from cows fed plantain swards can inhibit this process.

Two overseas studies have been published which incorporated plantain in a pasture and determined N_2O emissions and N dynamics in the soil (Bracken *et al.*, 2020; Pijlman *et al.*, 2019). Pijlman *et al.* (2019) found that the potential of nitrification was reduced with increasing proportion of plantain content in a ryegrass pasture, however, there was not an effect on soil NO_3^- concentration. A reduction of 39% in N_2O fluxes was reported when plantain was included, but there was no relationship with the increasing proportion of plantain in the pasture (Pijlman *et al.*, 2019). Bracken *et al.* (2020) observed that a multispecies sward containing plantain potentially inhibited the nitrification process.

Reductions in NO_3^- leaching have been reported when plantain was incorporated into diverse swards (Carlton *et al*., 2018; Malcolm *et al.*, 2014; Welten *et al*., 2019; Woods *et al*., 2018). When plantain comprised less than 10% of the swards, there were not reductions in NO_3^- leached over two seasons (Malcolm *et al.*, 2014). Carlton *et al*. (2018) found significant reduction in NO_3^- leaching from urine patches in a pasture containing plantain (ranging from 20-30%) compared to ryegrass-white clover swards. They observed a low abundance of AOB under plantain pastures, suggesting that plantain releases BNI which slow down the nitrification process. The AOB has been shown to drive the nitrification process in the soil when urine is applied, directly affecting the N_2O emissions and NO_3^- leaching from soils (Di *et al.*, 2009). However, Podolyan *et al.* (2019) did not observe differences in the abundance of AOB between plantain and ryegrass-white clover swards. The reason for this contrary result could be the season when the experiments were conducted. The first study was in summer and the last one in winter.

Differences in temperature could affect the growth of the AOB. In a mesocosm experiment, it was found that pure plantain reduced the potential of nitrification by 40% compared with perennial ryegrass, although no differences in NO_3^- leached were found among treatments (Pijlman *et al.*, 2019).

From all the studies reported above it seems that plantain swards have the potential to inhibit the nitrification process, however, there is still no clear mechanism. Research suggested that aucubin could be released in the root exudates of plantain. To our knowledge, only one experiment has studied the release of the secondary metabolites by the root system of plantain (Wurst *et al.*, 2010). Wurst *et al.* (2010) reported that when plantain was exposed to soil microorganisms and nematodes, aucubin and catalpol were excreted by the root system. The exudation of these metabolites is a defense mechanism and the presence of soil microorganisms enhanced aucubin concentration in roots (Wurst *et al.*, 2010).

2.5 CONCLUSIONS AND RESEARCH OBJECTIVES

The main conclusions drawn from this literature review are:

- Considerable short-term research has been undertaken on the effect of including plantain in the cows' diet, with urine concentration, urine volume and frequency, faecal N and the effect on milk yield all measured. Reduction in UNc has been reported from cows grazing diverse swards containing plantain compared to ryegrass-white clover swards due to a dilution effect on the urine of cows grazing plantain, increasing the frequency of urination. A reduction in UNc could lead to decreased NO_3^- leaching from urine patches. Further research is required to determine the effect of plantain on NO_3^- leaching at paddock scale when cows are grazing year-round in the paddocks. The scale of this study will allow the effect of the area affected by urine patches to be considered.

- It is known that environmental and soil conditions highly influence N_2O emissions. To date, small number of published data show the effect of plantain sward on N_2O emissions, and more data are needed comparing the effect of plantain swards under different seasons. To our knowledge, there are no data on the effect of plantain swards and urine from cows fed plantain, on NH_3 volatilisation.

- Earlier studies have suggested that plantain could excrete secondary metabolites via the root system into the soil, inhibiting the nitrification process. To date, there is only one paper that determined that soil microorganisms induce the exudation of these compounds by plantain swards. However, there are no published data on the release of these compounds by the roots when not induced by damage. Also, a small number of studies suggested that these compounds could be excreted in the cows' urine, however, no research has measured this.

- Aucubin, a secondary metabolite excreted by plantain, has been identified as a potential BNI, reducing N_2O emissions from urine patches. However, the effect of aucubin on NO_3^- leaching under urine patches has not been studied.

The **focus and objectives** for the three experimental chapters in this thesis are:

i. To determine the effect of plantain swards on the NO_3^- leaching at a large plot scale from three pasture treatments (plantain, plantain-clovers mix and ryegrass-white clover swards) grazed with dairy cows for three years: 2017, 2018 and 2019 (Chapter 3).

ii. To evaluate the effect of pure plantain sward and urine from cows fed plantain on N_2O and NH_3 emissions, and mineral N in soils during spring 2017 and late autumn/early winter 2018 (Chapter 4).

iii. To determine the effect of aucubin application on N_2O emissions and NO_3^- leaching from lysimeters collected from pure plantain and ryegrass-white clover swards when urine from animals offered the same diet was applied to the lysimeters (Chapter 5).

iv. To compare N_2O emissions and NO_3^- leaching when urine with the same N load from cows fed plantain and ryegrass-white clover, was applied to ryegrass-white clover swards (Chapter 5).

v. To examine the concentration of the secondary metabolites, aucubin and catalpol, in plantain root exudates (Chapter 5).

This thesis will contribute to current knowledge by studying the effect of plantain swards on cows' urine nitrogen concentration, milk production and NO_3^- leaching losses at a paddock scale. It will add data on the effect of plantain on N_2O and NH_3 losses under poor drainage soils and will determine if the effect on N losses is due to the excretion of the secondary metabolites by the roots of plantain.

2.6 REFERENCES

Aber, J. D. & Melillo, J. M. (1982). Nitrogen immobilization in decaying hardwood leaf litter as a function of initial nitrogen and lignin content. *Canadian Journal of Botany, 60*(11), 2263-2269.

Al-Mamun, M., Abe, D., Kofujita, H., Tamura, Y. & Sano, H. (2008). Comparison of the bioactive components of the ecotypes and cultivars of plantain (*Plantago lanceolata* L.) herbs. *Animal Science Journal, 79*(1), 83-88.

Al-Marashdeh, O., Judson, H., Schetelat, S., Pawsey, A. & Edwards, G. (2019). Urinary nitrogen excretion and water balance of dairy heifers fed cut plantain or perennial-ryegrass pastures. *New Zealand Journal of Animal Science and Production, 79,* 171-173.

Arnold, P. W. (1954). Losses of nitrous oxide from soil. *Journal of Soil Science, 5*(1), 116-128.

Bartholomaeus, A. & Ahokas, J. (1995). Inhibition of P-450 by aucubin: is the biological activity of aucubin due to its glutaraldehyde-like aglycone? *Toxicology Letters*, *80*, 75-83.

Berenbaum, M. & Rosenthal, G. A. (1992). Herbivores, Their Interactions with Secondary Plant Metabolites. Academic Press, San Diego, USA.

Betteridge, K., Costall, D. A., Li, F. Y., Luo, D. & Ganesh, S. (2013). Why we need to know what and where cows are urinating–a urine sensor to improve nitrogen models. In *Proceedings of the New Zealand Grassland Association*, *75,* 119-24.

Bol, R., Petersen, S. O., Christofides, C., Dittert, K. & Hansen, M. N. (2004). Short-term N_2O, CO_2, NH_3 fluxes, and N/C transfers in a Danish grass-clover pasture after simulated urine deposition in autumn. *Journal of Plant Nutrition and Soil Science*, *167*, 568-76.

Bolan, N. S., Saggar, S., Luo, J., Bhandral, R. & Singh, J. (2004). Gaseous emissions of nitrogen from grazed pastures: processes, measurements and modeling, environmental implications, and mitigation. *Advances in agronomy, 84,* 37-120.

Bourgaud, F., Gravot, A., Milesi, S. & Gontier, E. (2001). Production of plant secondary metabolites: a historical perspective. *Plant Science*, *161,* 839-51.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2017). Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research*, *60,* 470-82.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2019). Seasonal and diurnal changes in aucubin, catalpol and acteoside concentration of plantain herbage grown at high and low N fertiliser inputs. *New Zealand Journal of Agricultural Research*, *62*(3), 343-353.

Box, L.A. & Judson, H.G. (2018). The concentration of bioactive compounds in Plantago lanceolata is genotype specific. *Journal of New Zealand Grassland*, *80,* 113- 18.

Bracken, C. J., Lanigan, G. J., Richards, K. G., Müller, C., Tracy, S. R., Grant, J., Krol, D. J., Sheridan, H., Lynch, M. B., Grace, C., Fritch, R. & Murphy, P. N. C. (2020). Sward composition and soil moisture conditions affect nitrous oxide emissions and soil nitrogen dynamics following urea-nitrogen application. *Science of The Total Environment*, *722,* 137780.

Bryant, R. H., Snow, V. O., Shorten, P. R., & Welten, B. G. (2020) Can alternative forages substantially reduce N leaching? findings from a review and associated modelling. *New Zealand Journal of Agricultural Research, 63*:1, 3-28.

Bryant, R. H., Welten, B. G., Costall, D., Shorten, P. R. & Edwards, G. R. (2018). Milk yield and urinary-nitrogen excretion of dairy cows grazing forb pasture mixtures designed to reduce nitrogen leaching. *Livestock Science*, *209*, 46-53.

Buckthought, L. E., Clough, T. J., Cameron, K. C., Di, H. J. & Shepherd, M. A. (2015). Urine patch and fertiliser N interaction: Effects of fertiliser rate and season of urine application on nitrate leaching and pasture N uptake. *Agriculture, Ecosystems & Environment*, *203,* 19-28.

Butterbach-Bahl, K., Baggs, E. M., Dannenmann, M., Kiese, R. & Zechmeister-Boltenstern, S. (2013). Nitrous oxide emissions from soils: how well do we understand the processes and their controls? *Philosophical Transactions of the Royal Society B: Biological Sciences, 368*(1621), 20130122.

Cameron, K. C., Di, H. J. & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology*, *162,* 145-73.

Carlton, A. J., Cameron, K. C., Di, H. J., Edwards, G. R. & Clough, T. J. (2018). Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. *New Zealand Journal of Agricultural Research, 62* (2)*,* 150-172.

Carter, M. S. (2007). Contribution of nitrification and denitrification to N_2O emissions from urine patches. *Soil Biology and Biochemistry*, *39*(8), 2091-2102.

Castillo, A., Kebreab, E., Beever, D. & Ja, F. (2000). A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *Journal of Animal and Feed Sciences, 9*, 1-32.

Charlton, J. F. & Stewart, A. V. (1999). Pasture species and cultivars used in New Zealand – a list. In *Proceedings of the New Zealand Grassland Association*, 147-66.

Cheng, L. , Judson, H. G., Bryant, R. H., Mowat, H., Guinot, L., Hague, H., Taylor, S. & Edwards, G. R. (2017a). The effects of feeding cut plantain and perennial ryegrass-white clover pasture on dairy heifer feed and water intake, apparent nutrient digestibility and nitrogen excretion in urine. *Animal Feed Science and Technology*, *229,* 43-46.

Cheng, L., Mccormick, J., Hussein, A., Logan, C., Pacheco, D., Hodge, M. & Edwards, G. (2017b). Live weight gain, urinary nitrogen excretion and urination behaviour of dairy heifers grazing pasture, chicory and plantain. *The Journal of Agricultural Science*, *155,* 669-78.

Christensen, C., Hedley, M. J., Hanly, J. A. & Horne, D. J. (2018). Duration-controlled grazing of dairy cows. 2: nitrogen losses in sub-surface drainage water and surface runoff. *New Zealand Journal of Agricultural Research, 62*(1), 48-68.

Christensen, Christine Lynne. (2013). Duration-controlled grazing of dairy cows: impacts on pasture production and losses of nutrients and faecal microbes to water. Massey University, Palmerston North, New Zealand.

Constantinides, M. & Fownes, J. H. (1994). Nitrogen mineralization from leaves and litter of tropical plants: Relationship to nitrogen, lignin and soluble polyphenol concentrations. *Soil Biology and Biochemistry, 26*(1), 49-55.

Davidson, E. A. & Verchot, L. V. (2000). Testing the Hole-in-the-Pipe Model of nitric and nitrous oxide emissions from soils using the TRAGNET Database. *Global Biogeochemical Cycles*, *14*(4), 1035-1043.

Davini, E., Javarone, C., Trogolo, C., Aureli, P. & Pasolini, B. (1986). The quantitative isolation and antimicrobial activity of the aglycone of aucubin. *Phytochemistry*, *25,* 2420-22.

de Klein, C. A. M. (2001). An analysis of environmental and economic implications of nil and restricted grazing systems designed to reduce nitrate leaching from New Zealand dairy farms. II. Pasture production and cost/benefit analysis. *New Zealand Journal of Agricultural Research*, *44*, 217-35.

de Klein, C. A. M. & Eckard, R. J. (2008). Targeted technologies for nitrous oxide abatement from animal agriculture. *Animal Production Science*, *48,* 14-20.

de Klein, C. A. M., Barton, L., Sherlock, R. R., Li, Z. & Littlejohn, R. P. (2003). Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. *Soil Research*, *41*, 381-99.

de Klein, C. A. M. & Ledgard, S. F. (2001). An analysis of environmental and economic implications of nil and restricted grazing systems designed to reduce nitrate leaching from New Zealand dairy farms. I. Nitrogen losses. *New Zealand Journal of Agricultural Research*, *44*(2-3), 201-215.

de Klein, C. A. M., Luo, J., Woodward, K. B., Styles, T., Wise, B., Lindsey, S. & Cox, N. (2014). The effect of nitrogen concentration in synthetic cattle urine on nitrous oxide emissions. *Agriculture, Ecosystems & Environment*, 1*88,* 85-92.

de Klein, C. A. M., Smith, L. C. & Monaghan, R. M. (2006). Restricted autumn grazing to reduce nitrous oxide emissions from dairy pastures in Southland, New Zealand. *Agriculture, Ecosystems & Environment*, *112,* 192-99.

de Klein, C. A. M., van der Weerden, T. J., Luo, J., Cameron, K. C. & Di, H. J. (2019). A review of plant options for mitigating nitrous oxide emissions from pasture-based systems. *New Zealand Journal of Agricultural Research, 63*(1), 29-43.

Decau, M. L., Simon, J. C. & Jacquet, A. (2003). Fate of Urine Nitrogen in Three Soils throughout a Grazing Season. *Journal of environmental quality*, *32* (4), 1405-13.

Di, H. & Cameron, K, C. (2016). Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: a review. *Journal of Soils and Sediments*, *16*, 1401-20.

Di, H. & Cameron, K. C. (2002a). Nitrate leaching and pasture production from different nitrogen sources on a shallow stoney soil under flood-irrigated dairy pasture. *Soil Research*, *40*, 317-34.

Di, H. & Cameron, K. C. (2002b). Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems*, *64,* 237-56.

Di, H. & Cameron, K.C. (2002c). The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. *Soil Use and Management*, *18,* 395-403.

Di, H., Cameron, K. C., Podolyan, A., Edwards, G. R., de Klein, C. A. M., Dynes, R. & Woods, R. (2016). The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. *Journal of Soils and Sediments*, *16*, 2252-62.

Di, H., Cameron, K. C., Shen, J. P., Winefield, C. S., O'callaghan, M., Bowatte, S. & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience, 2*, 621-624

Dietz, M., Machill, S., Hoffmann, H. C. & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil*, *368,* 445-58.

Dijkstra, J., Oenema, O., Van Groenigen, J. W., Spek, J. W., Van Vuuren, A. M. & Bannink, A. (2013). Diet effects on urine composition of cattle and N_2O emissions. *Animal*, *7,* 292-302.

Dillon, P., Roche, J., Shalloo, L. & Horan, B. (2005). Optimising financial return from grazing in temperate pastures. In: Murphy JJ, editor. Proceedings of a satellite workshop of the XXth international grassland congress; p. 131–147.

Dodd, M., Dalley, D., Wims, C., Elliott, D. & Griffin, A. (2018). A comparison of temperate pasture species mixtures selected to increase dairy cow production and reduce urinary nitrogen excretion. *New Zealand Journal of Agricultural Research*: *62*(4), 504-527.

Edwards, G., Bryant, R., Smith, N., Hague, H., Taylor, S., Ferris, A. & Farrell, L. (2015). Milk production and urination behaviour of dairy cows grazing diverse and simple pastures. In *Proceedings of the New Zealand Society of Animal Production*, 79-83.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019a). Efficacy of aucubin as a nitrification inhibitor assessed in two Canterbury field trials. *New Zealand Journal of Agricultural Research*, *63*(1),73-86.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019b). Ruminant urine patch nitrification and N_2O flux: effects of urine aucubin rate in a laboratory trial. *New Zealand Journal of Agricultural Research*, *63*(1), 65-72.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J., Edwards, G. R. & de Klein, C. A. M. (2017). Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. *New Zealand Journal of Agricultural Research*, *61* (4) 495-503.

Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R. & de Klein, C.A.M. (2016). Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N_2O emissions: a review. *New Zealand Journal of Agricultural Research*, *59*(3), 301-317.

Haynes, R. J. & Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in agronomy*, *49,* 119-99.

Houlbrooke, D. J. (2005). A study of the quality of artificial drainage under intensive dairy farming and the improved management of farm dairy effluent using 'deferred irrigation'. Massey University, Palmerston North, New Zealand.

Houlbrooke, D. J., Horne, D. J., Hedley, M. J., Hanly, J. A. & Snow, V. O. (2003). The impact of intensive dairy farming on the leaching losses of nitrogen and phosphorus from a mole and pipe drained soil. Paper presented at the *Proceedings of the New Zealand Grassland Association, 65,* 179- 184.

Judson, H. G. & Edwards, G. R. (2016). Urinary nitrogen concentration from dairy heifers grazing kale supplemented with either plantain or perennial ryegrass baleage in winter. *Journal of New Zealand Grasslands*, *78,* 99-102.

Judson, H. G., Fraser, P. M. & Peterson, M. E. (2019). Nitrification inhibition by urine from cattle consuming *Plantago lanceolata. Journal of New Zealand Grasslands*, *81,* 11-116.

Kebreab, E., France, J., Beever, D. E. & Castillo, A. R. (2001). Nitrogen pollution by dairy cows and its mitigation by dietary manipulation. *Nutrient Cycling in Agroecosystems, 60*(1), 275-285.

Kyamanywa, N., Tait, I. M., Mitchell, C. M., Hedley, M. J., Pacheco, D. & Bishop, P. (2020). Effect of a late summer diet change from pasture to brassica crop and silages on dairy cow milk production and urinary nitrogen excretion. *New Zealand Journal of Agricultural Research*.

Ledgard, S. , Welten, B. & Betteridge, K. (2015). Salt as a mitigation option for decreasing nitrogen leaching losses from grazed pastures. *Journal of the Science of Food and Agriculture*, *95*, 3033-40.

Ledgard, S., Schils, R., Erikson, J. & Luo, J. (2009). Environmental impacts of grazed clover/grass pastures. In.: Teagasc, Oak Park, Carlow, Ireland.

Lee, J. M., Hemmingson, N. R, Minnee, E. M. K. C. & Clark E. F.(2015). Management strategies for chicory (*Cichorium intybus*) and plantain (*Plantago lanceolata*): impact on dry matter yield, nutritive characteristics and plant density. *Crop and Pasture Science, 66*(2):168-183

Leip, A., Billen, G., Garnier, J., Grizzetti, B., Lassaletta, L., Reis, S., Simpson, D., Sutton, M. A., de Vries, W., Weiss, F. & Westhoek, H. (2015). Impacts of European livestock production: nitrogen, sulphur, phosphorus and greenhouse gas emissions, land-use, water eutrophication and biodiversity. *Environmental Research Letters, 10*(11), 115004.

Luo, J., Balvert, S. F., Wise, B., Welten, B., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. & Judge, A. (2018). Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. *Science of The Total Environment*, *610–611*, 1271-80.

Malcolm, B. J., Cameron, K. C., Di, H. J., Edwards, G. R. & Moir, J. L. (2014). The effect of four different pasture species compositions on nitrate leaching losses under high N loading. *Soil Use and Management*, *30*, 58-68.

Mangwe, M. C., Bryant, R. H., Beck, M. R., Beale, N., Bunt, C. & Gregorini, P. (2019). Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. *Animal Feed Science and Technology*, *252*, 11-22.

Mangwe, M. C., Bryant, R. H., Beck, M. R., Fleming, A. E. & Gregorini, P. (2020). Grazed chicory, plantain or ryegrass–white clover alters milk yield and fatty acid composition of late-lactating dairy cows. *Animal Production Science*, *60*, 107-13.

Martin, K. E. (2018). Effects of nitrogen fertiliser and regrowth interval on herbage dry matter yield, chemical composition and nitrogen solubility of alternative pasture forages in irrigated Canterbury conditions. Lincoln University, New Zealand.

Mazzetto, A., Falconer, S., & Ledgard, S. (2021). Mapping the carbon footprint of milk for dairy cows. https://www.dairynz.co.nz/media/5794083/mapping-the-carbon-footprint-of-milk-for-dairy-cows-report-updated.pdf

McLaren, R. G. & Cameron, K. C. (1996). *Soil Science: Sustainable production and environmental protection* (Auckland, New Zealand).

Ministry for Primary Industries. (2013). https://www.mpi.govt.nz/news-and-resources/media-releases/dcd-update-testing-confirms-expectations-of-dcd-distribution-in-products/

Ministry for the Environment. (2018). New Zealand's greenhouse gas inventory 1990–2016. Fulfilling reporting requirements under the United Nations Framework Convention on Climate Change and the Kyoto Protocol. New Zealand

Government.(http://www.mfe.govt.nz/sites/default/files/media/Climate%20Change/National%20GHG%20Inventory%20Report%201990-2016-final.pdf).

Minnée, E. M. K., Kuhn-Sherlock, B., Pinxterhuis, J. B. and Chapman, D. F. (2019) "Meta-analyses comparing the nutritional composition of perennial ryegrass (Lolium perenne) and plantain (Plantago lanceolata) pastures. *Journal of New Zealand Grasslands 81*: 117-123.

Minnée, E. M. K., Leach, C. M. T. & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science*, *239*, 104093.

Minnée, E. M. K., Waghorn, G. C., Lee, J. M. & Clark, C. E. F. (2017). Including chicory or plantain in a perennial ryegrass/white clover-based diet of dairy cattle in late lactation: Feed intake, milk production and rumen digestion. *Animal Feed Science and Technology, 227,* 52-61.

Misselbrook, T. H., Powell, J. M., Broderick, G. A. & Grabber, J. H. (2005). Dietary Manipulation in Dairy Cattle: Laboratory Experiments to Assess the Influence on Ammonia Emissions. *Journal of Dairy Science*, *88,* 1765-77.

Moir, J. L., Cameron, K. C., Di, H. J. & Fertsak, U. (2011). The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system. *The Journal of Agricultural Science*, *149*, 473-85.

Moller, S. N., Parker, W. J. & Edwards, N.J. (1996). Within-year variation in pasture quality has implications for dairy cow nutrition. In *Proceedings of the New Zealand Grassland Association, 57,* 173-177.

Moorhead, A. J. & Piggot, G. J. (2009). The performance of pasture mixes containing 'Ceres Tonic' plantain (Plantago lanceolata) in Northland. In *Proceedings of the New Zealand Grassland Association, 71,* 195-199.

Navarrete, S. (2015). Evaluation of herb pastures for New Zealand dairy systems. Massey University, Palmerston North, New Zealand.

Navarrete, S., Kemp, P. D., Pain, S. J. & Back, P. J. (2016). Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata* L.) and their effect on *in vitro* rumen fermentation. *Animal Feed Science and Technology*, *222,* 158-67.

Navarrete, S., Kemp, P. D., Rodriguez, M. J., Horne, D. J., Hanly, J. A. & Hedley, M. J. (2020). Plantain (Plantago lanceolata) nitrogen use and excretion by lactating dairy cows. In Nutrient Management in Farmed Landscapes. (Eds C.L

Christensen, D.J.Horne and R.Singh). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 33. Farmed Landscapes Research Centre, Massey University, Palmerston North, New Zealand. 9 pages.

Nobilly, F., Bryant, R. H., McKenzie, B. A. & Edwards, G. R. (2013). Productivity of rotationally grazed simple and diverse pasture mixtures under irrigation in Canterbury. In *Proceedings of the New Zealand Grassland Association*, 165-72.

O'Connell, C.A., Judson, H. G. & Barrell, G. K. (2016). Sustained diuretic effect of plantain when ingested by sheep. In *Proceedings of the New Zealand Society of Animal Production*, 14-17. Adelaide.

Oenema, O., Velthof, G. L., Yamulki, G. L. & Jarvis, S. C. (1997). Nitrous oxide emissions from grazed grassland. *Soil Use and Management*, *13*, 288-95.

Olmos Colmenero, J. J. & Broderick, G. A. (2006). Effect of Dietary Crude Protein Concentration on Milk Production and Nitrogen Utilization in Lactating Dairy Cows1. *Journal of Dairy Science*, *89,* 1704-12.

Pacheco, D. & Waghorn, G. C. (2008). Dietary nitrogen–definitions, digestion, excretion and consequences of excess for grazing ruminants. In *Proceedings of the New Zealand Grassland Association*, 107-16.

Pakrou, N. & Dillon, P. J. (2004). Leaching losses of N under grazed irrigated and non-irrigated pastures. *The Journal of Agricultural Science*, *142*(5), 503-516.

Pijlman, J., Berger, S. J., Lexmond, F., Bloem, J., van Groenigen, J. W., Visser, E. J. W., Erisman, J. W. & van Eekeren, N. (2019). Can the presence of plantain (Plantago lanceolata L.) improve nitrogen cycling of dairy grassland systems on peat soils? *New Zealand Journal of Agricultural Research: 63*(1), 106-122.

Podolyan, A., Di, H. J. & Cameron, K. C. (2019). Effect of plantain on nitrous oxide emissions and soil nitrification rate in pasture soil under a simulated urine patch in Canterbury, New Zealand. *Journal of Soils and Sediments, 20,* 1468-1479.

Powell, A. M., Kemp, P. D., Jaya, K. & Osborne, M. A. (2007). Establishment, growth and development of plantain and chicory under grazing. *Proceedings of the New Zealand Grassland Association*, *69,* 41-45.

Ravishankara, A. R., Daniel, J. S. & Portmann, R. W. (2009). Nitrous Oxide (N_20): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science*, *326*, 123-25.

Rodriguez, M. J., Saggar, S., Berben, P., Palmada, T., Lopez-Villalobos, N. & Pal, P. (2019). Use of a urease inhibitor to mitigate ammonia emissions from urine patches. *Environmental Technology*.

Rumball, W., Keogh, R. G., Lane, G.E., Miller, J. E. & Claydon, R. B. (1997). Grasslands Lancelot' plantain (*Plantago lanceolata* L.). *New Zealand Journal of Agricultural Research, 40*(3), 373-377.

Saggar, S., Bolan, N. S., Bhandral, R., Hedley, C. B. & Luo, J. (2004). A review of emissions of methane, ammonia, and nitrous oxide from animal excreta deposition and farm effluent application in grazed pastures. *New Zealand Journal of Agricultural Research*, *47*, 513-44.

Saggar, S., Bolan, N. S. & Singh, J. (2005). Economic and environmental impacts of increased nitrogen use in grazed pastures and role of inhibitors in mitigating nitrogen losses. I(3), 62-67.

Saggar, S., Jha, N., Deslippe, J., Bolan, N. S., Luo, J., Giltrap, D. L., Kim, D. G., Zaman, M. & Tillman, R. W. (2013). Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. *Science of The Total Environment, 465*, 173-95.

Selbie, D. R., Buckthought, L. E. & Shepherd, M. A. (2015). The Challenge of the Urine Patch for Managing Nitrogen in Grazed Pasture Systems. *Advances in agronomy*, *129*, 229-92.

Sharpley, A. N. & Syers, J. K. (1979). Loss of nitrogen and phosphorus in tile drainage as influenced by urea application and grazing animals. *New Zealand Journal of Agricultural Research*, *22,* 127-31.

Shepherd, M. & Carlson, W. T. (2018). Urine patch size and nitrogen load: effects on nitrogen uptake from the urine patch in plantain and ryegrass/white clover pastures. *Journal of New Zealand Grasslands*, *80,* 195-200.

Shepherd, M., Carlson, B. & Lucci, G. (2017). The effect of time of winter urine deposition on mineral nitrogen leaching and implications for grazed forage crops. *New Zealand Journal of Agricultural Research, 60,* 376-85.

Shepherd, M., Phillips, P. & Snow, V. (2011). The challenge of late summer urine patches in the Waikato region. In: Adding to the knowledge base for the nutrient manager. (Eds L.D. Currie and C L. Christensen). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 24. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. 8 pages.

Sherlock, R. R. & Goh, K. M. (1984). Dynamics of ammonia volatilization from simulated urine patches and aqueous urea applied to pasture I. Field experiments. *Fertilizer Research*, *5,* 181-95.

Silva, R. G., Cameron, K. C., Di, H. J. & Hendry, T. (1999). A lysimeter study of the impact of cow urine, dairy shed effluent, and nitrogen fertiliser on nitrate leaching. *Soil Research*, *37*, 357-70.

Simon, P. L., de Klein, C. A. M., Worth, W., Rutherford, A. J. & Dieckow, J. (2019). The efficacy of Plantago lanceolata for mitigating nitrous oxide emissions from cattle urine patches. *Science of The Total Environment*, *691*, 430-441.

Singh, Jagrati. 2007. The role of inhibitors in mitigating nitrogen losses from cattle urine and nitrogen fertiliser inputs in pastures. Massey University, Palmerston North, New Zealand.

Stark, C. & Richards, K. G. (2008). The continuing challenge of agricultural nitrogen loss to the environment in the context of global change and advancing research. *Dynamic Soil, Dynamic Plant, 2*, 1-12.

Stewart, A. V. (1996). Plantain (Plantago lanceolata) – a potential pasture species. Proceedings of the New Zealand Grassland Association *58,* 77-86.

Subbarao, G. V., Ito, O., Sahrawat, K. L., Berry, W. L., Nakahara, K., Ishikawa, T., Watanabe, T., Suenaga, K., Rondon, M. & Rao, I. M. (2006). Scope and Strategies for Regulation of Nitrification in Agricultural Systems—Challenges and Opportunities. *Critical Reviews in Plant Sciences*, *25*, 303-35.

Tamura, Y. & Nishibe, S. (2002). Changes in the Concentrations of Bioactive Compounds in Plantain Leaves. *Journal of Agricultural and Food Chemistry*, *50,* 2514-18.

Totty, V. K., Greenwood, S. L., Bryant, R. H. & Edwards, G. R. (2013). Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *Journal of Dairy Science*, *96*, 141-49.

Tozer, K., Cameron, C. & Thom, E. (2011). Pasture persistence: farmer observations and field measurements. Pasture Persistence. *Grassland Research and Practice Series 15*:25-30.

van Groenigen, J. W., Kuikman, P. J., de Groot, W. J. M. & Velthof, G. L. (2005). Nitrous oxide emission from urine-treated soil as influenced by urine composition and soil physical conditions. *Soil Biology and Biochemistry*, *37*(3), 463-473.

Welten, B. G., Ledgard, S. F., Judge, A. A., Sprosen, M. S., McGowan A. W. & Dexter, M. M. (2019). Efficacy of different temperate pasture species to reduce nitrogen leaching from cattle urine applied in different seasons: A soil lysimeter study. *Soil Use and Management, 35*(4), 653-663.

Whitehead, D. C. (1995). *Grassland nitrogen* (CAB international).

Whitehead, D. C. & Raistrick, N. (1991). Effects of some environmental factors on ammonia volatilization from simulated livestock urine applied to soil. *Biology and Fertility of Soils*, *11*(4), 279-284.

Whitehead, D. C. & Raistrick, N. (1993). The volatilization of ammonia from cattle urine applied to soils as influenced by soil properties. *Plant and Soil, 148*(1), 43-51.

Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J. & Clough, T. J. (2018). Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. *Grass and Forage Science, 73*(4), 878-887.

Woodward, S. L., Waghorn, G. C., Bryant, M. A. & Benton, A. (2012). Can diverse pasture mixtures reduce nitrogen losses? In: Jacobs, J.L. (Ed.), Australasian Dairy Science Symposium. Department of Primary Industries, Melbourne, Australia, pp. 463–464.

Woodward, S. L., Waghorn, G., Watkins, K. A. & Bryant, M. A. (2009). Feeding birdsfoot trefoil (Lotus corniculatus) reduces the environmental impacts of dairy farming. In *Proceedings of the New Zealand Society of Animal Production*, 179- 83. Canterbury, New Zealand.

World Health Organisation. (2011). Guidelines for drinking water quality. Geneva, Switzerland.

Wrage, N., Velthof, G. L., van Beusichem, M. L. & Oenema, O. (2001). Role of nitrifier denitrification in the production of nitrous oxide. *Soil Biology and Biochemistry*, *33*, 1723-32.

Wurst, S., Wagenaar, R., Biere, A. & van der Putten, W. H. (2010). Microorganisms and nematodes increase levels of secondary metabolites in roots and root exudates of Plantago lanceolata. *Plant and Soil*, *329,* 117-26.

Zaman, M. & Blennerhassett, J. D. (2010). Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. *Agriculture, Ecosystems & Environment*, *136*, 236-46.

Zaman, M. & Nguyen, M. L. (2012). How application timings of urease and nitrification inhibitors affect N losses from urine patches in pastoral system? *Agriculture, Ecosystems & Environment*, *156,* 37-48.

Zaman, M., Nguyen, M. L., Blennerhassett, J. D. & Quin, B. F. (2008). Reducing NH_3, N_2O and NO_3^- –N losses from a pasture soil with urease or nitrification inhibitors and elemental S-amended nitrogenous fertilizers. *Biology and Fertility of Soils*, *44,* 693-705.

Zaman, M., Saggar, S., Blennerhassett, J. D. & Singh, J. (2009). Effect of urease and nitrification inhibitors on N transformation, gaseous emissions of ammonia and nitrous oxide, pasture yield and N uptake in grazed pasture system. *Soil Biology and Biochemistry*, *41*, 1270-80.

Zaman, M., Zaman, S., Nguyen, M. L., Smith, T. J. & Nawaz, S. (2013). The effect of urease and nitrification inhibitors on ammonia and nitrous oxide emissions from simulated urine patches in pastoral system: A two-year study. *Science of The Total Environment*, *465,* 97-106.

3 NITRATE LEACHING LOSSES FROM PLANTAIN SWARDS

3.1 ABSTRACT

The incorporation of plantain (*Plantago lanceolata*) into pastures on dairy farms could be a potential mitigation strategy to reduce nitrogen (N) losses to the environment. This study evaluated the effect of grazing plantain on: urine-N and urea-N concentration in cows' urine, nitrate (NO_3^-) leaching losses and milk production. The experiment was conducted during two full grazing seasons (2017/2018 and 2018/2019). Three mobs of 20 cows were allocated to graze three pasture treatments: (i) plantain, (ii) plantain (70%) – clovers (30%) mix, (plantain, red [*Trifolium pratense*] and white clover [*T. repens*]), or (iii) ryegrass (*Lolium perenne*) (70%) – white clover (30%) mix. The pastures were established in a complete randomised design (n=5) in plots of 800 m^2 (40 m x 20 m) with isolated mole-pipe drain systems to collect drainage water from each plot treatments. The treatments were allocated to the plots according to a complete randomised design. In the 2018 drainage season, the NO_3^- leaching in pure plantain was reduced by 48 and 58% compared to ryegrass-white clover and plantain-clovers mix pastures, respectively. In the 2019 drainage season, NO_3^- leaching under the plantain and ryegrass-white clover swards were similar and lower than that under plantain-clovers mix swards. In the summer and autumn of both grazing seasons, the urine-N and urea-N concentrations were reduced in the urine of cows grazing pure plantain compared to urine from cows grazing ryegrass-white clover pastures. Cows grazing plantain and plantain-clovers mix pastures produced similar milk solids to the cows grazing ryegrass-white clover pastures. Therefore, plantain may be used as a mitigation strategy to reduce NO_3^- leaching so long as the plantain content in the pastures is greater than 30%.

Keywords: NO_3^- leaching, *Plantago lanceolata*, urine N concentration, milk production, lactating cows.

3.2 INTRODUCTION

The intensification and expansion of dairy farming in New Zealand has increased nitrogen (N) leaching losses, which has contributed to a deterioration in water quality. Nitrate (NO_3^-) concentrations exceeding 11.3 mg L^{-1} in drinking water present a human health risk (World health Organisation, 2011). In addition, high N concentrations in freshwater bodies can also result in eutrophication and algal blooms (Leip *et al.*, 2015). Regional councils across New Zealand are formulating Land and Water Plans and are often focused on developing regulations that limit the amount of N leached from agricultural soils.

Urine patches have been identified as the largest source of N leached from grazed pastures (Cameron *et al.*, 2013). The N loading rate in dairy cow urine can be as large as 2000 kg N ha^{-1} (Selbie *et al.*, 2015), but is more typically 400-800 kg N ha^{-1} (Cameron *et al.*, 2013; Haynes & Williams, 1993). The N concentration in urine patches exceeds the N requirements of plants and the surplus is prone to leaching (Cameron *et al.*, 2013; Haynes & Williams, 1993). Urine patches deposited during grazing over the period from late summer to early winter (late lactation), have been shown to have a major influence on the NO_3^- vulnerable to leaching during the winter drainage season (Shepherd *et al*. 2011, 2017). Therefore, the development of mitigation strategies that reduce the amount of NO_3^- accumulating in urine patches during the late lactation period are necessary for reducing NO_3^- losses from dairy systems to water bodies.

Recently, the inclusion of alternative forages such as plantain (*Plantago lanceolata* L.) into grass and legume pastures have been investigated as an option to reduce NO_3^- leaching from pastoral dairy systems. The inclusion of plantain into cows' diets has been shown to reduce urine N concentrations compared to perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pastures (Box *et al.*, 2016; Cheng *et al.*, 2017; Edwards *et al.*, 2015; Judson & Edwards, 2016; Minnée *et al.*, 2017, 2020; Totty *et al.*, 2013; Woodward *et al.*, 2012). This reduction in the N loading rate could potentially decrease the risk of NO_3^- leaching losses from urine patches.

In addition, there is evidence suggesting that plantain can produce specific secondary metabolites that may inhibit the nitrification process in the soil (Dietz *et al.*, 2013). A reduction in the rate of nitrification decreases the rate at which ammonium (NH_4^+) is converted into NO_3^-, reducing the accumulation of soil NO_3^-. A biological inhibition of nitrification is therefore a potential method by which plantain might reduce N losses from dairy systems to the environment.

Research related to the role of plantain reducing NO_3^- leaching from urine patches has focussed on lysimeters studies. A lysimeter study showed that the inclusion of plantain into pastures reduced NO_3^- leaching by 78% when the urine from cows grazing ryegrass-white clover pasture was applied (Carlton *et al.*, 2018). Carlton *et al.* (2018) also reported that the incorporation of plantain into a sward reduced the abundance of the soil ammonia oxidiser bacteria (AOB) compared to ryegrass-white clover soil, suggesting an inhibition of the nitrification process. Woods *et al.* (2018) found a 45% reduction in NO_3^- leaching when urine from cows fed on ryegrass-white clover was applied to lysimeters growing Italian ryegrass swards containing 42% of plantain. There is a need for large-scale plot studies to evaluate the use of plantain swards as a mitigation strategy to reduce NO_3^- leaching, while quantifying the effect of plantain swards on milk production.

The objective of this experiment was to determine the ability of plantain swards to mitigate NO_3^- leaching from grazed pasture. To achieve this objective a two-year grazing plot study was conducted to quantify the impacts of plantain swards on dairy pasture production, urine N concentrations of dairy cows, urea-N concentration in urine and milk yield and N losses in drainage water. It was hypothesised that: i) the incorporation of plantain into cows' diet will reduce NO_3^- leaching from paddocks; ii) urine-N and urea-N concentrations from cows fed pure plantain and plantain-clovers mix swards will be lower than concentration in urine from cows grazing ryegrass-white clover swards; and iii) milk production from cows fed on plantain or plantain-clovers mix will be similar than milk from cows fed on ryegrass-white clover.

3.3 MATERIALS AND METHODS

3.3.1 Experimental site and design

This field study was a paddock scale experiment which was set up at Massey University's Dairy Farm 4, near Palmerston North (40° 39' S; 175 ° 61' E). The soil type at the research site is Tokomaru silt loam, which is classified as an Argillic-fragic Perch-gley Pallic Soil (Hewitt, 2010). A detailed description of soil physical properties is provided by Scotter *et al*. (1979).

The area used for this study was subdivided into 15 plots. Each plot was 800 m^2 (40 m x 20 m), and had its own mole and pipe drain system. Mole channels were ploughed at 2 m intervals at 0.45 m depth. These channels were intercepted by a gravel backfilled trench overlying a perforated pipe drain (0.11 m diameter and installed at 0.60 m of depth). This drainage system was installed before the pasture treatments were established. Pasture preparation started in October 2016 following application of herbicide (80 L/ha) to the existing pastures. The pastures were sown in early summer (1st December 2016).

Three pasture treatments were evaluated: (i) plantain (100%), (ii) plantain-clovers mix, containing plantain (70%), red clover (*Trifolium pratense* L.) (15%) and white clover (*T. repens* L.) (15%), and (iii) perennial ryegrass-white clover mix (Table 3.1). There were five replicates of each treatments (15 plots in total) and the treatments were established using a complete randomised design (Figure 3.1).

The plots were grazed by lactating dairy cows (the management is described in Section 3.3.2) during March and April 2017 (establishment phase), from September 2017 to June 2018 (2017/2018 grazing season), and from September 2018 to May 2019 (2018/2019 grazing season). Two grazings were carried out at the beginning of the 2019/2020 grazing season, in September 2019 and October 2019 (Table 3.2). Three additional paddocks (approximately 1 ha) of each pasture treatment were also established near the experimental plots. These paddocks were used to transition and adapt the cows' diet to the pasture treatments (6 days) before grazing the experimental plots (2-3 days) (Figure 3.1).

Animal ethics approval for this experiment was granted by the Massey University Animal Ethics Committee, application No.: 16/137.

Table 3.1. Pasture treatments (plantain, plantain/clover mix and ryegrass-white clover pastures), cultivars and sowing rate (kg seed/ha) for each pasture.

Pasture	**Species and cultivars**	**Sowing rate (kg seed/ha)**
Plantain	Plantain cv. 'Tonic'	10
Plantain/clover mix	Plantain cv. 'Tonic'	8
	Red clover cv. 'Relish'	4
	White clover cv. 'Emerald'	4
Ryegrass- clover mix	Ryegrass cv. 'Trojan'	25
	White clover cv. 'Emerald'	3

Figure 3.1. Layout of the 15 experimental plots. ‘PL’ denotes plantain pasture, ‘Mix’ denotes plantain/clover mixed pasture and ‘RG’ denotes ryegrass-white clover pasture.

3.3.2 Grazing management

3.3.2.1 Establishment phase

There were two grazing events in the establishment phase (March and April 2017). In these two grazings, 36 lactating dairy cows were selected from the dairy farm herd and separated into three groups (n=12); one herd for each treatment. Cows were blocked into 3 groups according to milk solids production, days in milk and liveweight. After five days grazing in the adaptation paddocks, the cows were transferred to the experimental plots (experimental period). Each herd spent the next five days grazing the five plots of each treatment i.e. spending one day in each replicate plot.

3.3.2.2 Grazing seasons

After the establishment phase, there was a change in the management of the grazings of the plots. In 2017/2018, and 2018/2019 grazing seasons, 60 lactating dairy cows were selected from the dairy farm herd and separated into three groups (n=3 with 20 cows/group). Each herd grazed one of the three pastures treatments through the two grazing seasons. Following six days of grazing the adaptation paddocks, the cows were transferred to the plots (1.5-3 days). During the experimental period, the 15 plots were grazed simultaneously. The number of cows in each plot ranged from 3 to 4 cows, and the number of days they were on the plots varied from 1.5 to 3 days, depending on the target DM intake per cow. Plots were grazed by cows based on a pre-graze target cover of 2800-3300 kg DM ha^{-1}.

Cows were milked twice daily, at approximately 07:00 and 14:30, from September 2017 until November 2017, and from December 2017 to May 2018 once a day (OAD) (approx. 07:00). In the second season, cows were milked twice a day (approx. 07:00 and 15:30) from September 2018 until March 2019, and OAD in May 2019 (approx. 07:00).

Table 3.2 shows the plots' management during the establishment phase and both of the full grazing seasons.

Table 3.2. Grazing management of the three pasture treatments in each grazing period, number of cows into the plots, amount of supplements, the total amount of hours the cows grazed the plots and periods when the animal samplings were carried out.

Season	Grazing period	Grazing Date	Cows per plots	Supplements [1] (kg DM/cow/day)	Grazing hours on the plots	Animal sampling[2]
Establishment phase	Mar 2017		12	6	18	
	Apr 2017	19th to 28th Apr	12	5	18	
Season 2017-2018	Spring	27th to 29th Sep	4		18	X
	Late spring	1st to 3rd Nov	4		18	
	Early summer	5th to 7th Dec	4	6.5	21*	X
	Late summer	7th to 9th Feb	4	5	21*	X
	Autumn	7th to 9th Mar	5	7	21*	X
	Late autumn	2nd to 4th May	3	7	21*	
	Winter	13th to 14th Jun	3		22**	
Season 2018-2019	Early spring	19th to 21st Sep	3; 2.5 days	8.5	18	
	Spring	31st to 2nd Oct	3; 2.5 days	8	18	
	Late spring	28th to 30th Nov	4; 2.5 days	7	18	
	Early summer	18th to 20th Dec	4	6	18	X
	Summer	16th to 18th Jan	4	5	18	
	Late summer	13th to 15th Feb	4; 2.5 days	7	18	X
	Early autumn	26th to 27th Mar	4; 1.5 days	5.5	18	
	Autumn	7th to 8th May	4; 1.5 days	6	21*	X
	Sep 2019	10th to 12th	5			
	Oct 2019	15th to 17th	4			

[1] Supplements were Maize silage, Pasture baleage, Dairy pellet, and Dried Distillers Grains (DDG), tapioca; [2] Cows were sampled for urine and milk; *cows milked OAD; **Dry cows.

3.3.3 Pasture management

3.3.3.1 Establishment phase

The total experimental site was mowed on 10th of January 2017 due to an infestation of fathen (*Chenopodium album* L.). No N fertiliser was applied during the establishment phase.

3.3.3.2 Grazing seasons

In the 2017/2018 season, the three pasture treatments received 60 kg N/ha as AMMO31 (60:40, ammonium sulphate: urea, 31% N) split into two applications in August and October 2017. In January 2018, urea fertiliser was applied at 35 kg N ha^{-1} to the pasture treatments. The adaptation area and the plots were mowed after the grazing of November 2017; and after the grazing of December 2017, the ryegrass-white clover plots were mowed.

In August 2018 (during the 2018/2019 grazing season), ryegrass-white clover and pure plantain plots received 30 kg N ha^{-1} as urea. In October urea was applied at 50 kg N ha^{-1} to the plantain plots only. All the ryegrass-white clover plots were mowed in October 2018.

3.3.4 Measurements

3.3.4.1 Pasture measurement and cow intakes

Pre and post-grazing herbage mass (kg dry matter (DM) ha^{-1}) were determined by cutting three herbage samples to ground level (0.1 m^2 quadrat), randomly chosen from each plot, using an electric shearing handpiece. The pre-grazing samples were taken the day before the cows grazed the plots, and the post-grazing samples were cut immediately after grazing the plots. Each sample was washed to remove the soil and oven dried at 70 ^{o}C for 48 h. Samples for botanical composition were taken at the same time as the pre-grazing samples, by cutting a strip of pasture (50 x 10 cm) next to each herbage mass pre-grazing quadrat sample. The botanical composition was determined by separating the samples into the following categories: plantain, white and red clover; perennial ryegrass, broadleaf weeds and other grass and dead material, and oven-dried individually at 70 ^{o}C for 48h.

Then, the proportion of each category was calculated as a percentage of total sample DM weight.

For total N content of pastures, a sample (200 g fresh weight) of each plot was collected pre-grazing. The sample was obtained by hand plucking at multiple random spots across each plot. Total N (TN) was determined by combustion using a Leco analyser (AOAC, 2000; method 968.06).

Aucubin was measured in herbage from the plantain sward which was collected by taking a hand grab sample from multiple sites in pure plantain plots. During the establishment phase, the samples were collected at both grazing times, March and April 2017. During the first grazing season, herbage samples were taken on five dates: September and December 2017; February, March and May 2018. During the second grazing season, samples were taken on eight dates: September, October, November and December 2018; January, February, March and May 2019. Samples were dried at 70 ^{o} C and ground to pass through a 1 mm diameter sieve before analysis.

Aucubin in plantain leaves was determined by high-performance liquid chromatography (HPLC). A 100 mg quantity of each of the ground samples was taken for extraction with 10 mL of methanol (MeOH) in 15 mL tubes and shaken for 2 h at room temperature. The solid plant material was filtered out using grade 41 quantitative filter papers (Whatman Co., Ltd., England). A 2 mL aliquot of the filtrate was then diluted in 8 mL of ultra-pure water, and then further filtered using a 0.2 µm syringe filter (Whatman Co., Ltd., England). A 20 µL aliquot of this solution was used for HPLC analysis for the determination of aucubin. Commercially available aucubin, (99% pure; Extrasynthese S.A, France) was used as the standard.

The standard solution contained 2 mg of aucubin in 50 mL of 20% MeOH. High-performance liquid chromatography was performed at 40 ^{o}C using a 100 mm × 6.0 YMC pack ODS-A column protected by a YMC guard pack (YMC America, Inc). The mobile phase was 1% acetronitrile in water for aucubin. The flow rate was 1 mL/min. The wavelength detection was performed at 240 nm. The HPLC system consisted of a Dionex UltiMate 3000 HPLC system equipped with an UltiMate 3000 Pump, an UltiMate 3000

Autosampler Column Compartment, an UltiMate 3000 variable wavelength detector and Chromeleon software (version 6.8) for data processing.

3.3.4.2 Cow measurements

Individual cow milk yields (L/day) were automatically recorded during each grazing period (DeLavel Alpro Herd Management System, DeLavel, Tumba, Sweden). These results are shown as the average of the milk volume/day, per pasture treatment for the total time cows spent into the plots. Milk samples were collected from each cow during the milking on day 8 to analyse for milk solids (MS) content. In the 2017/2018 grazing season, milk solids content was determined in early spring 2017 and early/late summer 2018, and autumn 2018. In the 2018/2019 grazing season, milk solids content was determined in early/late spring 2018, late summer 2019 and autumn 2019 (Table 3). The cow's milk yields were recorded during those seasons because the main objective was to monitor if there was any change in milk production due to a pasture effect.

Spot urine samples were collected four times during the 2017/2018 and 2018/2019 grazing seasons (Table 3.2). Urine samples were collected from each cow after manual stimulation of the vulva. The urine samples were acidified below pH 4 with sulfuric acid 6 N (Normal) to avoid volatilisation and then frozen until analysed. Urine samples were collected immediately after the morning milking on days 7 and 8, to analyse for total N and urea-N concentration. Total N was determined by combustion using a Leco analyser (AOAC, 2000; method 968.06), and urea-N was analysed using the Urease Kinetic UV assay.

3.3.4.3 Drainage measurements and water analysis

This study involved monitoring drainage from the grazing plots over three drainage seasons; i.e. the 2017 drainage season (establishment phase), and the 2018 and 2019 grazing seasons. Drainage water from each plot was channelled through the collecting pipe into individual tipping-bucket (5 L) flow meters located in sampling pits nearby (Figure 3.2). The tipping buckets were instrumented with data loggers to provide continuous measurements of flow rate. During each drainage event, a proportion (ca. 0.1%) of the drainage water from every second tip of the tipping bucket flow meter was automatically collected to provide a volume-proportioned, mixed sample for analysis.

Approximately 100 mL was taken from the drainage water collected at the tipping bucket meter at each event. A subsample was taken for NO_3^- and total N analyses. About half of each subsample was filtered through a 0.45 um filter paper to analyse for NO_3^-, while total N (TN) was analysed from the unfiltered subsample. Nitrate was measured using the Ion Chromatography (Dionex Aquion). Total N was determined using the persulphate digestion method of Hosomi and Sudo (1986), in which the different forms of N in the sample are converted to NO_3^- and analysed using the colorimetric method described for NO_3^-.

3.3.4.4 Soil water balance and soil samples

A soil water balance (Scotter *et al*. 1979) was used to predict when drainage would occur. The Climate data were obtained from a weather station located on Dairy Farm 4.

Soil samples were taken in August 2018 and September 2019, to monitor the soil mineral N content in the plots. In August 2018, soil samples were collected from the 0-7.5 cm soil depth and in September 2019 samples were taken from 0-7.5 cm and 7.5-15 cm soil depths. A total of seven soil cores were collected from each plot and were combined together to provide a single bulk sample for each soil depth. A subsample of 3 g of field moist soil was extracted with 25 ml of 2M potassium chloride (KCl) to determine NO_3^- and ammonium (NH_4^+) concentrations colorimetrically using a Technicon Auto Analyser (Blakemore, 1987).

Figure 3.2. Individual tipping buckets for collection of drainage water in one of the collection shelters.

3.3.5 Calculations and Statistical analysis

The herbage mass that accumulated between grazing events was calculated as the pre-grazing herbage minus the post-grazing herbage mass of the previous grazing (Hodgson, 1979).

The apparent pasture DM intake (DMI) per cow during the total time the cows spent into the plots (experimental period) was calculated as the herbage disappearance between pre- and post-grazing:

Apparent DMI (kg DM/cow/days)*days on the plots =

[(pre-graze herbage mass – post-graze herbage mass (kg DM/ha) x plot area(ha)] / (cows/days) * days into the plots (3.1)

The apparent herbage N intake in the diet during the total time the cows spent into the plots (experimental period) was estimated by the following equation:

Apparent N intake (kg N/cow/days)*days on the plots=

[(DMI) × N % of pre-graze herbage × plot area (ha)]/

(cows/days) * days into the plots (3.2)

Data were analysed using the PROC MIXED procedure of SAS 9.4 (SAS Institute, 2009) using a model for a complete randomised design. Means were compared using the least squares means test and significance was declared at $P < 0.05$. The pasture accumulation during each grazing season was analysed using a model that included the fixed effect of pasture treatments (plantain, plantain-clovers mix and ryegrass-white clover pastures). Milk production, urine N concentration and urea-N in urine, NO_3^- and total N in water samples, were analysed using a model that included the fixed effects of pasture treatments and the replicates as the random effect.

3.4 RESULTS

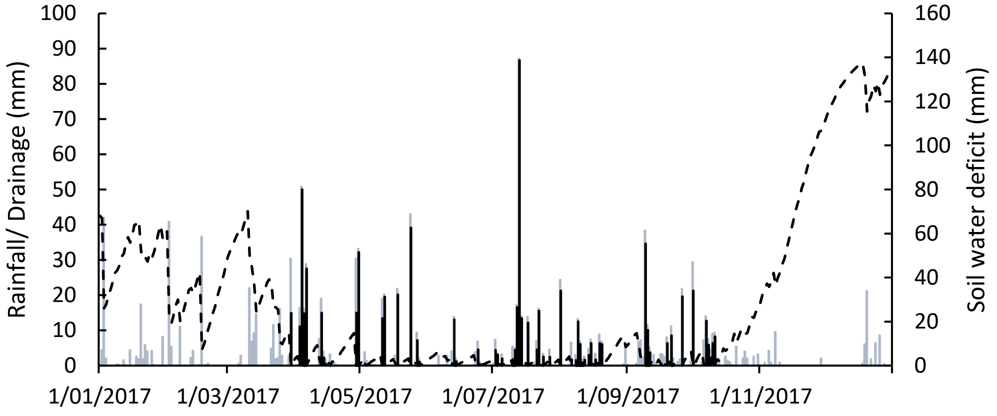
3.4.1 Climatic conditions and drainage

During each drainage season, drainage events coincided with a soil water deficit of zero as predicted by the soil water balance model (Figure 3.3) or when a heavy rainfall event occurred. During 2017, the establishment year, the total annual rainfall was 1300 mm. Average annual drainage depths (mm) for the three pasture treatments were not significantly different (*P*= 0.54). Drainage depths were 416 ± 16, 375 ± 30 and 372 ± 39 mm for the plantain, plantain-clovers mix and ryegrass-white clover pasture treatments, respectively. The first drainage event occurred in April and the last drainage event occurred in November (Figure 3.3a).

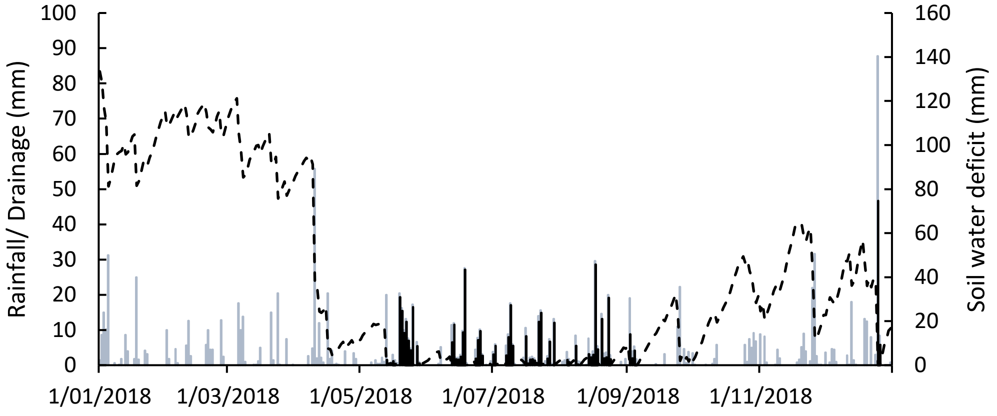
The total rainfall in 2018 was 1190 mm. The average annual drainage depths were similar (*P*= 0.69): 232 ± 9, 230 ± 10 and 213 ± 18 mm for plantain, plantain-clovers mix and ryegrass-white clover pasture treatments, respectively. The first drainage event was in May and the last drainage event occurred in December (Figure 3.3b).

The total rainfall between January and October in 2019 was 823 mm. In 2019, the drainage started in June and the last drainage event that was recorded was on 24th October. The decision was made to conclude the experiment at this point as it was a dry winter/spring period and little further drainage was expected. The cumulative drainage depths during this year were similar (*P*= 0.33): 184 ± 12, 155 ± 12 and 180 ± 17 mm drainage for plantain, plantain-clovers mix and ryegrass-white clover pastures, respectively (Figure 3.3c).

a) 2017 establishment year



b) 2018



c) 2019

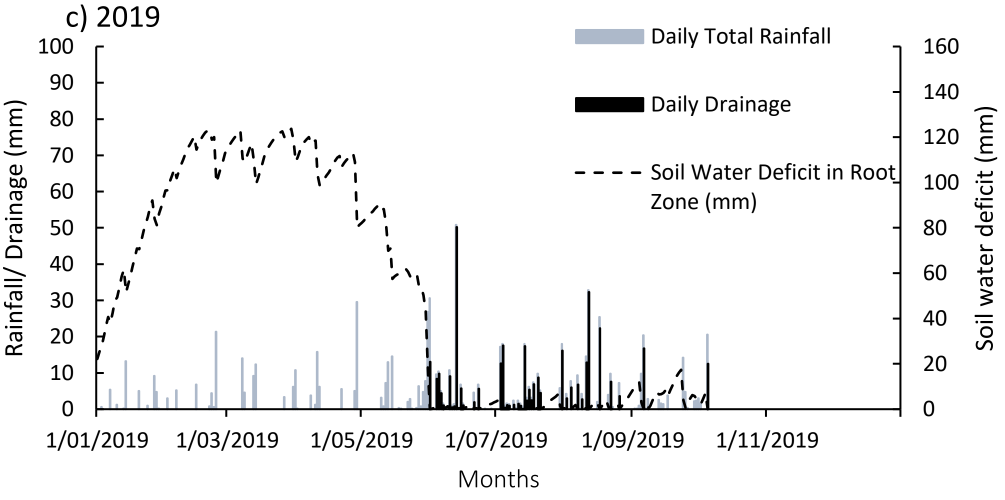


Figure 3.3. Rainfall (mm), soil water deficit (mm) and mean drainage depth (mm) during a) 2017 (establishment phase); b) 2018 and c) 2019 drainage seasons. Drainage depth and soil water deficit are absolute values. The data was obtained from a weather station near the experimental site.

3.4.2 Pasture accumulation, pasture and N intake, and botanical composition

Pasture accumulation during the establishment phase was similar (*P*= 0.24) among the three pasture treatments (Table 3.3). Pasture accumulation during the 2017/2018 season (i.e. between August 2017 and August 2018) were similar (*P*= 0.18), among the three pasture treatments (Table 3.3). In the 2018/2019 growing season (i.e. between August 2018 and August 2019), annual pasture accumulation was 25 and 29% greater (*P*= 0.05) for ryegrass-white clover (13524 ± 1128 kg DM ha^{-1}) and plantain-clovers mix (14180 ± 1342 kg DM ha^{-1}), respectively, compared to the plantain pasture treatment (10134 ± 703 kg DM ha^{-1}).

In the 2017/2018 grazing season, total apparent DM intakes for the experimental period were similar for the three treatments. In early spring, the apparent DM intake was greater for cows grazing ryegrass-white clover (*P*= 0.05) than plantain and plantain-clovers mix pastures. The total apparent N intake (Table 3.4) during the 2017/2018 grazing season was similar for cows fed plantain (3537 ± 537 g/cow) and ryegrass-white clover (3757 ± 379 g N/cow) pastures, and these were lower (*P*= 0.06) than N intake by cows fed plantain-clovers mix pastures (5239 ± 463 g N/cow). In early spring 2017 cows fed ryegrass-white cover tended to have greater (*P*= 0.06) N intake than plantain and plantain-clovers mix. In early summer 2018, cows fed plantain-clovers mix had greater (*P*= 0.06) N intake than plantain and ryegrass-white clover pastures.

In the 2018/2019 season, total apparent DM intakes for the experimental period tended to be greater (*P*= 0.06) for the plantain-clovers mix than for ryegrass-white clover and plantain. The total apparent N intakes (Table 3.4) were not statistically different between cows fed plantain (4130 ± 237 g/cow) and ryegrass-white clover (4783 ± 533 g N/cow) pastures, but N intakes for both of these pasture treatments were lower (*P* = 0.01) compared to the N intake by cows that grazed the plantain-clovers mix (6595 ± 648 g N/cow) pastures. There were also seasonal differences in N intake, with intakes being significantly higher for the plantain-clover mix, compared to the other two treatments, in early spring 2018 (*P*= 0.01) and early summer 2019 (*P*= 0.02).

The cows were offered supplementary feed according to the management practices of the Dairy Farm 4. The amount of supplements offered was the same for the three groups of cows (Table 3.2), therefore, no additional effect of the supplements on the cows' milk production was expected. The percentage of supplements eaten relative to the dry matter intake per cow during the experimental period is shown in Table 3.3. In the 2017/2018 grazing season, the proportion of supplements eaten ranged from 26 to 52%, 18 to 48% and 33 to 49% in ryegrass-white clover, plantain and plantain-clovers mix pastures, respectively. In the 2018/2019 grazing season, it ranged from 30 to 53%, 25 to 67% and 23 to 54% in ryegrass-white clover, plantain and plantain-clovers mix pastures, respectively.

Table 3.3. Pasture accumulation during the establishment phase, 2017/2018 and 2018/2019 seasons, and total apparent pasture intake (kg DM/cow/days into the plots) during the total time cows spent into the plots for the three pasture treatments, ryegrass/wc, plantain and plantain-clovers mix. (Values are Mean ± SEM).

	Pasture Treatment				
	Ryegrass/wc	**Plantain**	**Plantain-clovers mix**	**Number of days on plots**	***P* value**
Pasture removed (kg DM ha^{-1})					
Establishment phase	3946 ± 239	4129 ± 359	4720 ± 350		0.24
Season 1 (2017-2018)	13378± 980	12832 ± 492	14395 ± 1047		0.18
Season 2 (2018-2019)	13524 ± 1128a	10134 ± 703b	14180 ± 1342a		0.05
Total apparent pasture intake during the experimental period (kg DM/cow)					
Season 1(2017-2018)					
Early spring 2017	34 ± 3.0^{a}	25 ± 3.5^{b}	23 ± 1.0^{b}	2	0.05
Late spring 2017	29 ± 6.3 (52%)*	36 ± 2.9 (30%)	29 ± 6.9 (43%)	2	0.74
Early summer 2018	18 ± 3.9 (44%)	22 ± 2.5 (38%)	30 ± 5.7 (33%)	2	0.24
Late summer 2018	n.v	n.v	n.v	2	
Early autumn 2018	17 ± 4.5(50%)	19 ± 4.8 (48%)	27 ± 6.1 (38%)	2	0.24
Late autumn 2018	25 ± 7.1 (49%)	29 ± 7.7 (48%)	25 ± 6.4 (49%)	2	0.92
Early winter 2018	16 ± 3.1	15 ± 6.5	20 ± 9.0	1	0.96
*Total DMI during expt. Period (kg DM/Cow)**	*170 ± 22*	*185 ± 26*	*186 ± 22*		0.68
Season 2 (2018-2019)					
Early spring 2018	29 ± 6 (48%)	26 ± 5 (49%)	31 ± 5 (43%)	2.5	0.63
Late spring 2018	21 ± 7 (48%)	25 ± 2 (43%)	31 ± 3 (33%)	2.5	0.49
Early summer 2019	27 ± 4.5 (33%)	17 ± 4.4 (50%)	30 ± 6.3 (33%)	2	0.20
Summer 2019	31 ± 3.9 (31%)	21 ± 3.2 (44%)	32 ± 6.4 (38%)	2.5	0.11
Early autumn 2019	21 ± 3.9 (30%)	27 ± 4.1 (25%)	24 ± 4.2 (28%)	1.5	0.63
Late autumn 2019	12 ± 5.9 (53%)	13 ± 3.5 (67%)	14 ± 5.9 (54%)	1.5	0.65
*Total DMI during expt. Period (kg DM/Cow)***	*212 ± 22* ^{ab}	*164 ±11*^{b}	*245 ± 27*^{a}		0.06

n.v: no value; * numbers in brackets are the percentage of supplements in the cows' diets; $^{a\text{-}c}$ Means within a row with different superscripts letter are significantly different (*P*< 0.05); *the sum of the apparent DMI per cow during seven grazing carried out in 2017/2018 during the total time that cows were into the plots; **the sum of the apparent DMI per cow during eight grazing carried out in 2018/2019 during the total time that cows were into the plots.

Table 3.4. Apparent nitrogen (N) intake (g N/cow/days on the plots) by cows in each full grazing season (2017/2018 and 2018/2019) during the total time cows spent on the plots for the three pasture treatments. (Values are Mean ± SEM).

	Pasture Treatment				
	Ryegrass/wc	**Plantain**	**Plantain-clovers mix**	**Number of days on plots**	***P* value**
Apparent N Intake during experimental period (g N /Cow)					
Season 1(2017-2018)					
Early spring 2017	972 ± 153^{a}	600 ± 89^{b}	581 ± 47^{b}	2	0.06
Late spring 2017	1182 ± 454	1153 ± 135	1283 ± 351	2	0.47
Early summer 2018	570 ± 46^{b}	540 ± 76^{b}	1036 ± 149^{a}	2	0.06
Late summer 2018	n.v	n.v	n.v	2	
Early autumn 2018	582 ± 160	631± 172	1065 ± 177	2	0.24
Late autumn 2018	675 ± 184	766 ± 251	791 ± 211	2	0.42
*Total N intake during expt. Period (g N /Cow)**	*3757± 379^{b}*	*3537 ± 537^{b}*	*5239 ± 463^{a}*		0.06
Season 2(2018-2019)					
Early Spring 2018	965 ± 196	760 ± 139	964 ± 134	2.5	0.64
Late Spring 2018	568 ±150^{b}	574 ± 97^{b}	1468 ± 243^{a}	2.5	0.01
Early summer 2019	797± 140^{b}	413 ± 114^{b}	1106 ± 234^{a}	2	0.02
Summer 2019	953 ± 140	578 ±114	682 ± 234	2.5	0.48
Early autumn 2019	538 ± 100	841 ± 135	876 ± 157	1.5	0.24
Late autumn 2019	425 ± 167	325 ± 96	535 ± 241	1.5	0.63
*Total N intake during expt. Period (g N /Cow)***	*4783 ± 533^{b}*	*4130 ± 237^{b}*	*6595 ± 648^{a}*		*0.01*

$^{a-c}$ Means within a row with different superscripts letter are significantly different ($P < 0.05$);); *the sum of the apparent N intake per cow during the seven grazing carried out in 2017/2018, during the total time that cows were into the plots; **the sum of the apparent DMI per cow during the eight grazing carried out in 2018/2019, during the total time that cows were into the plots.

Table 3.5. Nitrogen (N) concentration (%) in ryegrass-white clover (Ryegrass/wc) plantain and plantain-clovers mix pastures during both grazing seasons, season 1 (2017/2018) and season 2 (2018/2019). (Values are Mean ± SEM).

	Pasture treatment			
	Ryegrass/wc	**Plantain**	**Plantain-clovers mix**	***P* value**
% of N in the sward				
Season 1 (2017/2018)				
Early spring 2017	2.72 ± 0.09	2.42± 0.10	2.56 ±0.21	0.37
Late spring 2017	2.78±0.04	2.64± 0.22	2.58± 0.30	0.80
Early summer 2018	3.16±0.11^{b}	2.48± 0.08^{c}	3.48± 0.10^{a}	<0.0001
Late summer 2018	2.96±0.07^{a}	2.46 ± 0.20^{b}	2.76± 0.13^{a}	0.04
Early autumn 2018	3.42±0.19	3.22± 0.17	3.54± 0.14	0.42
Late autumn 2018	2.72±0.06^{b}	2.63 ± 0.07^{b}	3.00 ±0.14^{a}	0.009
Season 2 (2018-2019)				
Early spring 2018	3.38± 0.05	3.12 ±0.17	3.44± 019	0.31
Late spring 2018	2.68±0.19	2.4±0.15	2.84±0.12	0.18
Early summer 2019	2.98± 0.11^{b}	2.84±0.14^{b}	3.76± 0.10^{a}	0.0003
Late summer 2019	2.22± 0.13	2.48±0.15	2.68± 0.12	0.06
Early autumn 2019	2.54± 0.09^{c}	3.14±0.09^{b}	3.56± 0.10^{a}	<0.0001
Late autumn 2019	3.27± 0.20^{b}	3.6±0.09^{ab}	3.74± 0.14^{a}	0.06

Table 3.5 shows the N concentration (%) of the pastures during the grazing events. On five of the six occasions that significant difference were observed in N concentrations of the swards, the plantain-clovers mix had the greatest N concentrations. During the 2017/2018 grazing season, in early summer the N concentration was higher (*P*< 0.0001) in plantain-clovers mix than in pure plantain and ryegrass-white clover pastures. In late summer 2018, N concentration was similar in plantain-clovers mix and ryegrass-white clover which were greater (*P*= 0.04) than pure plantain pastures. In early summer of 2019, the N concentrations in plantain-clovers mix was greater (*P*= 0.0003) than pure plantain and ryegrass-white clover pastures. In early autumn, the pastures with the lowest (*P*< 0.0001) N concentration was ryegrass-white clover while plantain-clovers mix had the highest N concentration.

The botanical composition of the three pasture treatments is presented in Figure 3.4. During the 2017/2018 grazing season, the proportion of ryegrass in the ryegrass-white clover pastures changed through the season ranging from 33 to 57%. The proportion of white clover was low and ranged from 2% to 11%. In the 2018/2019 grazing season, the proportion of ryegrass decreased through the grazing season from 58 to 26%; and clover content ranged from 12% to 14%.

In the plantain pasture treatment, during the 2017/2018 grazing season, the proportion of plantain decreased from 54% in spring 2017 to 24% in early summer 2018, increasing to 50% in late autumn 2018. The other components of this pasture were white clover, weeds and dead material. The proportion of white clover was low and constant through the season and ranged from 3 to 7%. During the 2018/2019 grazing season, the proportion of plantain was 64% in spring 2018, decreasing to 28% of the pasture in late autumn 2019. The white clover content increased during this season, ranging from 9 to 17%.

During the 2017/2018 grazing season, the plantain-clovers mix pasture treatment was comprised of 50% plantain in spring, decreasing in early and late summer 2018 to 24 and 29%, respectively. In late autumn 2018, the proportion of plantain was 45%. The proportion of red clover increased through the grazing season, ranging from 10 to 30% of the pasture. The white clover content was low throughout the season. In the 2018/2019 grazing season, the plantain content in plantain-clovers mix pastures decreased over the grazing season, from 61 to 15%. The proportion of red clover increased from 13 to 35%, and then decreased to 18% in late autumn 2019.

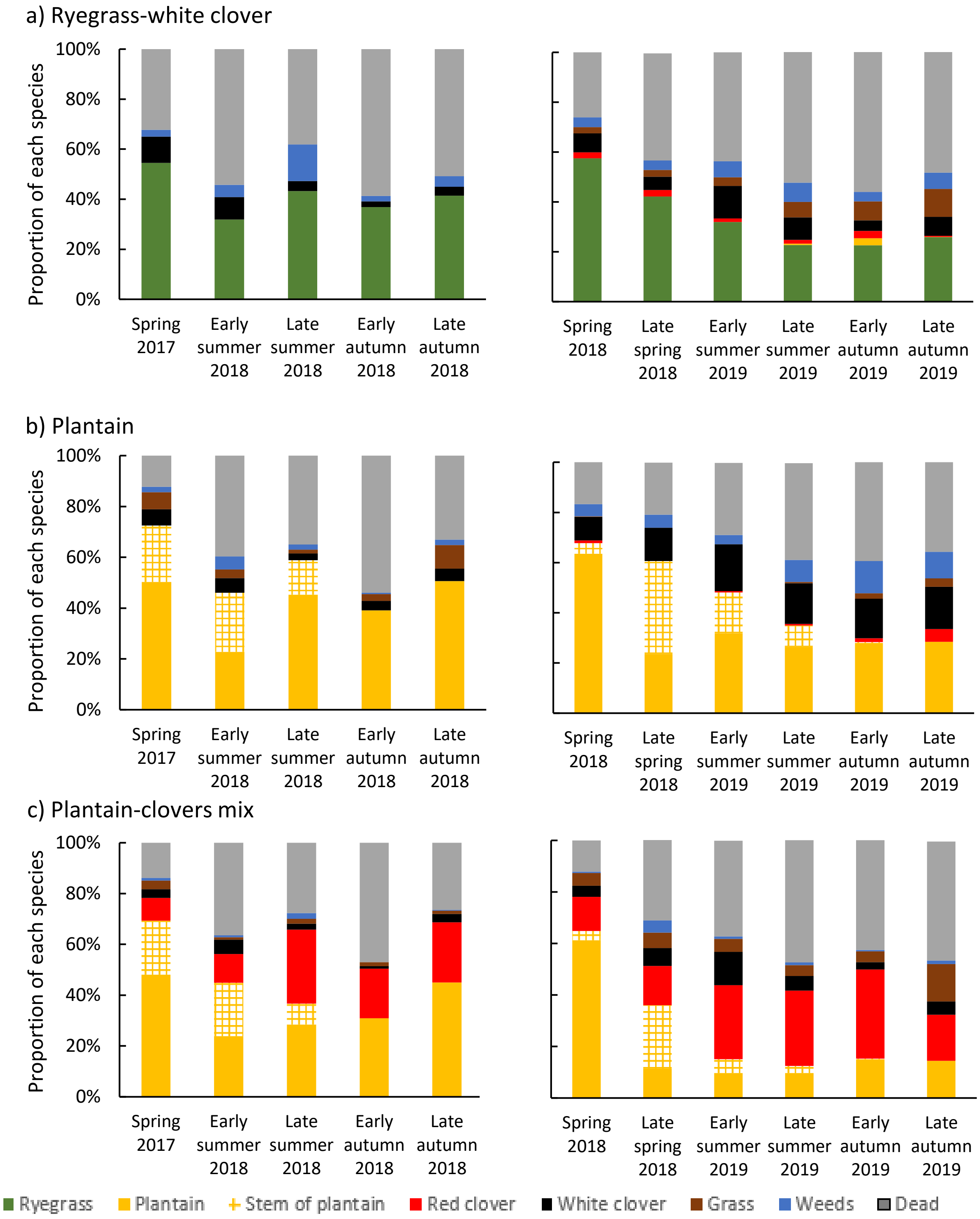


Figure 3.4. Botanical composition (%) in a) Ryegrass-white clover; b) plantain and c) plantain-clovers mix pastures during season 2017/2018 (left) and 2018/2019 (right).

Of the three pasture treatments, the plantain-clover mix pasture treatment maintained the highest clover contents over the two seasons (Figure 3.4). The clover content in the plantain pasture treatment increased in the second season, and this treatment had more clover than the ryegrass-white clover pasture treatment.

3.4.3 Urinary nitrogen

During the 2017/2018 grazing season, an effect of pasture treatments on urine N concentration (UNc) was observed in late summer and autumn (Table 3.6). In late summer, UNc in urine from cows fed plantain (2.90 g L^{-1}) and plantain-clovers mix (3.62 g L^{-1}) were 31 and 14% lower compared to urine from cows fed ryegrass-white clover (4.20 g L^{-1}), respectively. In autumn, UNc in urine from cows fed plantain (3.35 g L^{-1}) and plantain-clovers mix (3.82 g L^{-1}) were 40 and 35% lower compared to urine from cows fed ryegrass-white clover (5.57 g L^{-1}) urine, respectively. The relatively low UNc in urine from cows grazing plantain clover mix is striking given the large N intake by these cows. The urea-N concentration in urine was significantly ($P< 0.001$) affected by pasture treatments in early spring 2017, and late summer and autumn 2018. In early spring 2017, urea-N in urine from cows fed plantain was 37 and 11% lower, compared to urine from cows fed ryegrass-white clover and plantain-clovers mix pastures, respectively. In late summer and autumn 2018, the urea-N content in the urine of cows fed plantain (1.57 and 1.98 g L^{-1}, respectively) was 39 and 43% lower compared to urine from cows fed ryegrass-white clover (2.59 and 3.45 g L^{-1}, respectively). In late summer 2018, the urea-N content in urine of cows fed plantain (1.57 g L^{-1}) was 22% lower than urine of cows fed plantain-clovers mix pastures (2.00 g L^{-1}).

In the 2018/2019 grazing season, pasture treatments affected UNc and urea-N concentrations in urine in late summer and autumn. In late summer, UNc and urea-N concentrations in urine from cows fed plantain were 23% and 40% lower ($P= 0.010$ and $P= 0.001$, respectively), compared to urine from cows fed plantain-clovers mix, respectively, and 22 and 30% lower than for the ryegrass-white clover treatment, respectively. In autumn, the UNc and urea-N concentrations in urine from cows fed plantain were 23 and 17% lower ($P= 0.010$ and $P= 0.001$, respectively), compared to urine from cows fed plantain-clovers mix, respectively, and 16 and 22% lower than for the ryegrass-white clover treatment, respectively .

Table 3.6. Mean daily urinary nitrogen (N) concentration (g L^{-1}) and urea-N (g L^{-1}) for cows grazing ryegrass-white clover (Ryegrass/wc), plantain and plantain-clovers mix pasture treatments during 2017/2018 and 2018/2019. (Values are Mean ± SEM).

	Pasture Treatment			
	Ryegrass/wc	**Plantain**	**Plantain-clovers mix**	***P* value**
N urine (g L^{-1})				
Season 1(2017-2018)				
Early spring 2017	3.18 ± 0.24	2.85 ± 0.25	2.95± 0.27	0.61
Early summer 2018	4.40 ± 0.25	3.93 ± 0.34	4.09 ± 0.31	0.31
Late summer 2018	4.20 ± 0.30^{a}	2.90 ± 0.35^{b}	3.62 ± 0.26^{b}	0.001
Autumn 2018	5.57 ± 0.39^{a}	3.35 ± 0.38^{b}	3.82 ± 0.28^{b}	<.0001
Season 2 (2018-2019)				
Early spring 2018	Nd	nd	nd	
Early summer 2019	4.14 ± 0.25	4.14 ± 0.26	4.22 ± 0.27	0.84
Late summer 2019	5.79 ± 0.38^{a}	4.48 ± 0.45^{b}	5.85 ±0.67^{a}	0.01
Autumn 2019	7.42 ± 0.54^{a}	6.22 ± 0.49^{b}	8.08 ± 0.59^{a}	0.01
Urea-N in urine (g L^{-1})				
Season 1(2017-2018)				
Early spring 2017	1.22 ± 0.13^{a}	0.77 ± 0.09^{b}	1.09 ± 0.12^{a}	0.001
Early summer 2018	2.90 ± 0.16	2.83 ± 0.23	2.99 ± 0.23	0.73
Late summer 2018	2.59 ± 0.19^{a}	1.57 ± 0.14^{c}	2.00 ± 0.16^{b}	<.0001
Autumn 2018	3.45 ± 0.25^{a}	1.98 ± 0.22^{b}	2.41 ± 0.17^{b}	<.0001
Season 2 (2018-2019)				
Early summer 2019	3.58 ± 0.24	3.29 ± 0.20	4.09 ± 0.28	0.09
Late summer 2019	3.36 ± 0.28^{a}	2.34 ± 0.23^{b}	3.91 ± 0.42^{a}	0.001
Autumn 2019	5.99 ± 0.29^{a}	4.67 ± 0.30^{b}	5.64 ± 0.75^{a}	0.02

$^{a\text{-}c}$ Means within a row with different superscripts letter are significantly different ($P< 0.05$); nd: no determined.

3.4.4 Aucubin concentration in plantain pastures

During the establishment phase, aucubin concentrations in pure plantain swards increased from 0.5 ± 0.4 mg g^{-1} in March 2017 to 6.6 ± 0.4 mg g^{-1} in April 2017 (Figure 3.5). In the 2017/2018 grazing season, aucubin concentrations in plantain were similar in September 2017 (6.5 ± 0.4 mg g^{-1}) and in February 2018 (7.0 ± 0.5 mg g^{-1}). Aucubin concentrations then decreased sharply in autumn 2018, with a concentration of 2.8 ± 1.3

mg g^{-1} in March 2018 and 3.7 ± 0.7 mg g^{-1} in May. In the 2018/2019 season, aucubin concentrations was 4.2 ± 1.1 mg g^{-1} in September, and 0.4 ± 0.3 mg g^{-1} in December 2018, peaking in February 2019 at 6.8 ± 0.8 mg g^{-1}.

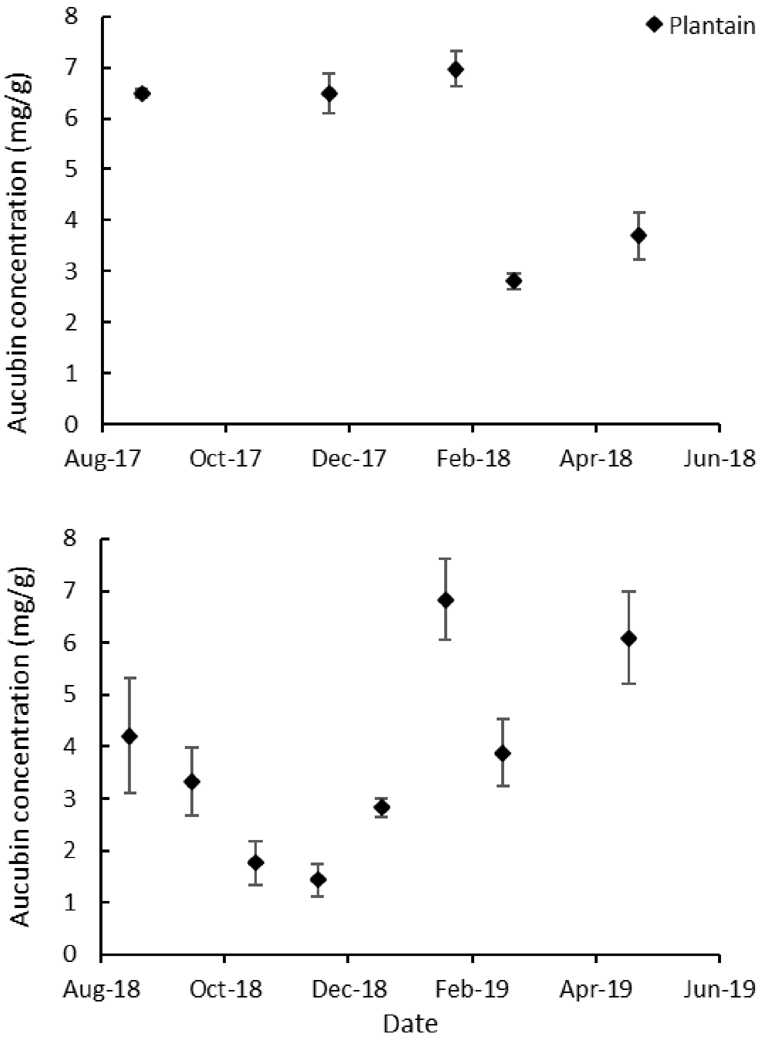


Figure 3.5. Aucubin concentration (mg g^{-1} DM) in plantain during the 2017/2018 season, and 2018/2019 season.

3.4.5 Milk production and milk solids

During the 2017/2018 grazing season, the milk yield (L) during the grazing of the experimental plots was only affected by pasture treatments in late summer 2018 (*P*= 0.008). The cows grazing in the plantain-clover mix treatment produced a higher volume of milk compared to the ryegrass-white clover treatment, and milk volume from cows grazing plantain was similar to cows grazing plantain-clovers mix and ryegrass-white clover (Table 3.7). In the 2018/2019 grazing season, cows in the ryegrass-white clover and plantain-clover mix treatments produced a larger volume of milk (*P*= 0.03) than the plantain pastures in early spring 2018. However, in late spring 2018, early summer 2019 and late summer 2019, the milk volumes produced by cows fed plantain and plantain-clovers mix pastures were similar, and higher than the ryegrass-white clover treatment. The MS production from cows during the experimental period (kg MS/cow) was similar among the three pasture treatments in both seasons, except for early spring 2017 where the MS production was similar from cows grazing ryegrass-white clover and plantain-clovers mix, which was greater (*P*= 0.04) than for cows grazing plantain.

Table 3.7. Milk yield (litres of milk produced per cow during the total time the cows spent into the plot) and milk solids (MS) production (kg MS during the total time the cows spent into the plot), for the 2017/2018 and 2018/2019 seasons. (Values are Mean ± SEM).

	Pasture Treatment				
	Ryegrass/wc	**Plantain**	**Plantain-clovers mix**	**Number of days on plots**	***P* value**
Milk yield (L/cow/during the experimental period)*					
Season 1 (2017/2018)					
Early spring 2017	53.1± 2.2	46.2 ± 1.7	47.6 ± 3.4	2	0.14
Late spring 2017	58.4 ± 2.3	56.7 ± 1.6	58.1 ± 1.7	2	0.80
Early summer 2018	43.7 ± 2.0	42.3 ± 1.4	42.5 ± 1.4	2	0.72
Late summer 2018	30.0 ±1.0^{b}	32.7 ± 0.9^{ab}	34.7 ± 1.2^{a}	2	0.008
Early autumn 2018	27.0 ± 0.7	25.8 ± 0.8	27.5 ± 1.1	2	0.34
Late autumn 2018	34.2 ± 3.0	25.6 ± 3.5	25.4 ± 3.9	2	0.18
Season 2 (2018/2019)					
Early spring 2018	102.2± 3.9^{a}	97.5 ± 2.8^{b}	101.3 ± 3.8^{a}	2.5	0.03
Late Spring 2018	79.1 ± 4.7^{b}	89.3± 4.5^{a}	88.2 ± 3.3^{ab}	2.5	0.02
Early summer 2019	70.3 ± 1.5^{b}	78.0 ± 2.0^{a}	76.0 ± 1.9^{a}	2	0.009
Late summer 2019	50.5 ± 1.2^{b}	59.4 ± 1.1^{a}	58.1 ± 1.6^{a}	2.5	<.0001
Early autumn	20.7 ± 1.1^{b}	23.1± 1.0^{ab}	25.3 ±1.3^{a}	1.5	0.02
Late Autumn	13.9± 2.0	18.0 ±10	16.4± 2.0	1.5	0.28
Milk solids production during experimental period (kg MS/cow/during the exp. Period)*					
Season 1(2017/2018)					
Early spring	6.0 ± 0.2^{a}	5.6 ± 0.1^{b}	6.1± 0.2^{a}	2	0.04
Early summer (Dec)	5.7 ± 0.5	5.1 ± 0.3	4.7 ±0.3	2	0.16
Late summer (Feb)	4.4± 0.2	4.6 ±0.3	4.9 ±0.2	2	0.09
Late autumn (May)	3.9 ±0.4	3.5± 0.4	3.9 ±0.5	2	0.79
Season 2(2018/2019)					
Early spring (Sep)	6.1 ± 0.3	5.8 ± 0.4	6.1 ± 0.3	2.5	0.78
Late spring (Nov)	4.9 ± 0.2	5.1 ± 0.08	4.9 ± 0.2	2.5	0.67
Late summer (Feb)	3.4 ± 0.1	3.6 ± 0.1	3.7± 0.2	2.5	0.31
Late autumn (May)	1.5 ± 0.2	1.7 ± 0.07	1.6 ± 0.2	1.5	0.85

$^{a-c}$ Means within a row with different superscripts letter are significantly different ($P< 0.05$); * the total milk production per season (L/cows and MS production) was not estimated due to an imbalance of the number of cows per season.

3.4.6 Nitrogen losses in drainage water

In the three years of this study, there was a general trend of a high NO_3^--N concentration in the drainage water at the start of each drainage season. This was followed by a steady decrease in NO_3^--N concentration in drainage through the rest of the season (Figure 3.6) except for the increase at the end of 2018, mainly in plantain and plantain-clovers mix treatments. In 2017, the cumulative NO_3^- for all treatments were very low as the treatments were only recently established. In both the 2018 and 2019 drainage seasons, there was one drainage event in November and December, respectively, at which NO_3^--N concentration in the drainage water increased for the three pasture treatments. The largest quantity of NO_3^- was lost in the first 50 to 100 mm of drainage depth in all the pasture treatments regardless of the drainage season (Figure 3.6).

In 2018, the average cumulative NO_3^- loss from the plantain (2.8 ± 0.4 kg NO_3^--N ha^{-1}) treatment was 58 and 48% lower (P= 0.002) than the losses from the plantain-clovers mix (6.6 ± 0.4 NO_3^--N ha^{-1}) and ryegrass-white clover (5.4 ± 0.8 kg NO_3^--N ha^{-1}) treatment, respectively. In 2019, the highest NO_3^- loss was observed for the plantain-clover mix (9.5 ± 1.0 kg NO_3^--N ha^{-1}) treatment plots, which was greater (P= 0.0003) than the plantain (4.0 ± 0.3 kg NO_3^--N ha^{-1}) and ryegrass-white clover (3.1 ± 0.4 kg NO_3^--N ha^{-1}) treatment. The plantain treatment was 57% lower than the plantain-clover mix treatment and not significantly different from the ryegrass-white clover pasture treatment. The NO_3^- leaching losses for the ryegrass-white clover treatment was 67% lower than the plantain-clovers mix pastures.

The concentration of TN in the drainage water (Figure 3.7) during the three years exhibited similar patterns to those observed for NO_3^- leaching. These patterns are expected as NO_3^- is a large component of TN (Table 3.8).

Soil samples collected in August 2018 and September 2019 did not show any effect of pasture treatments on mineral N (Table 3.9). These results were expected because the urine patches only cover a small area and 7 soil cores were taken for each paddock. Soil samples were taken in late winter/early spring when the mineral N concentration in the soil is typically low.

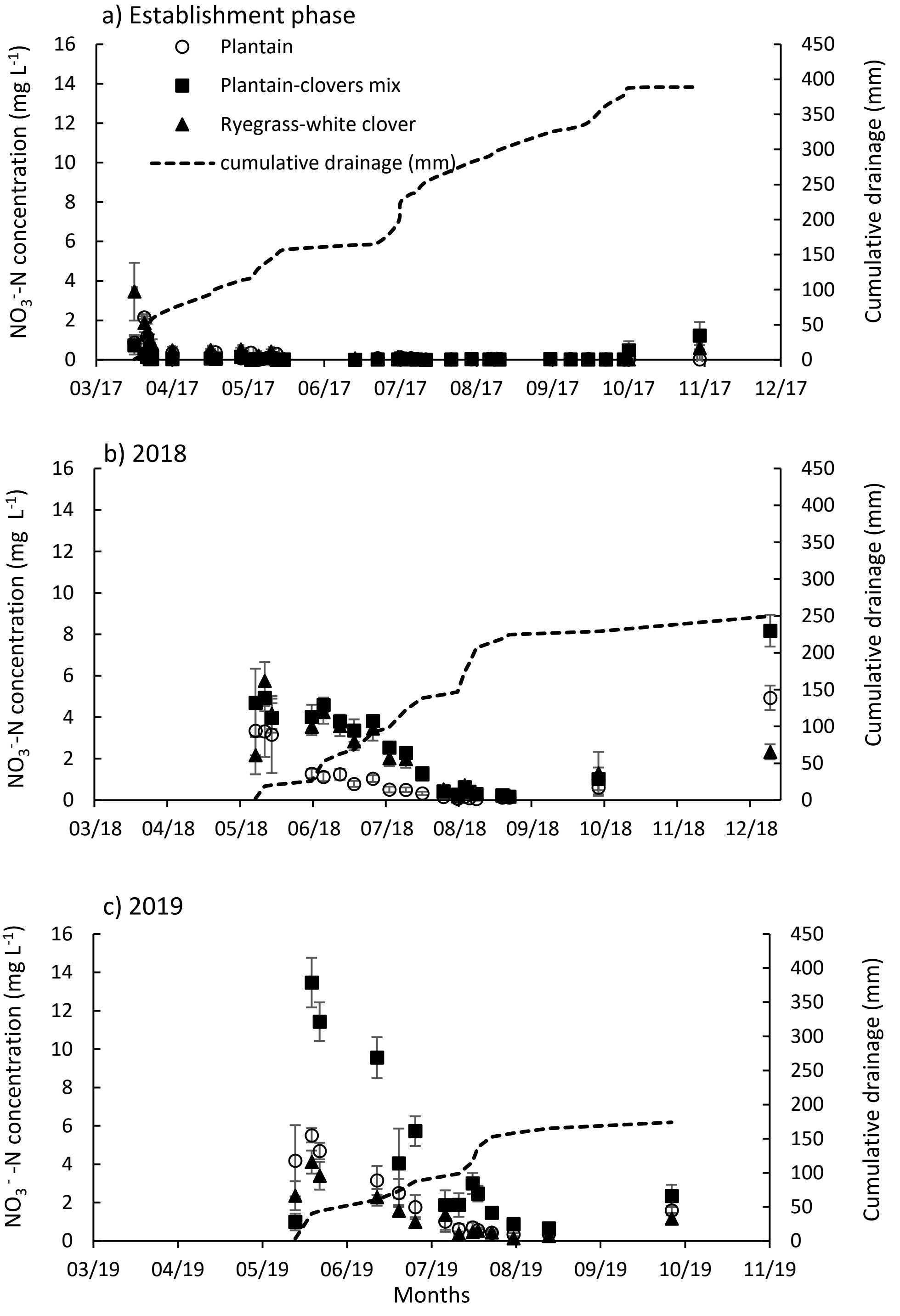


Figure 3.6. Nitrate concentration (mg NO_3^--N L^{-1}) in mole-pipe drainage water in a) the establishment phase, b) 2018 season and c) 2019 season, and the cumulative depths of drainage (mm) (dash line) for pure plantain (white circle), plantain-clovers mix (black square) and ryegrass- white clover (black triangle) pastures. Error bars represent the SEM at each sampling event (n=5).

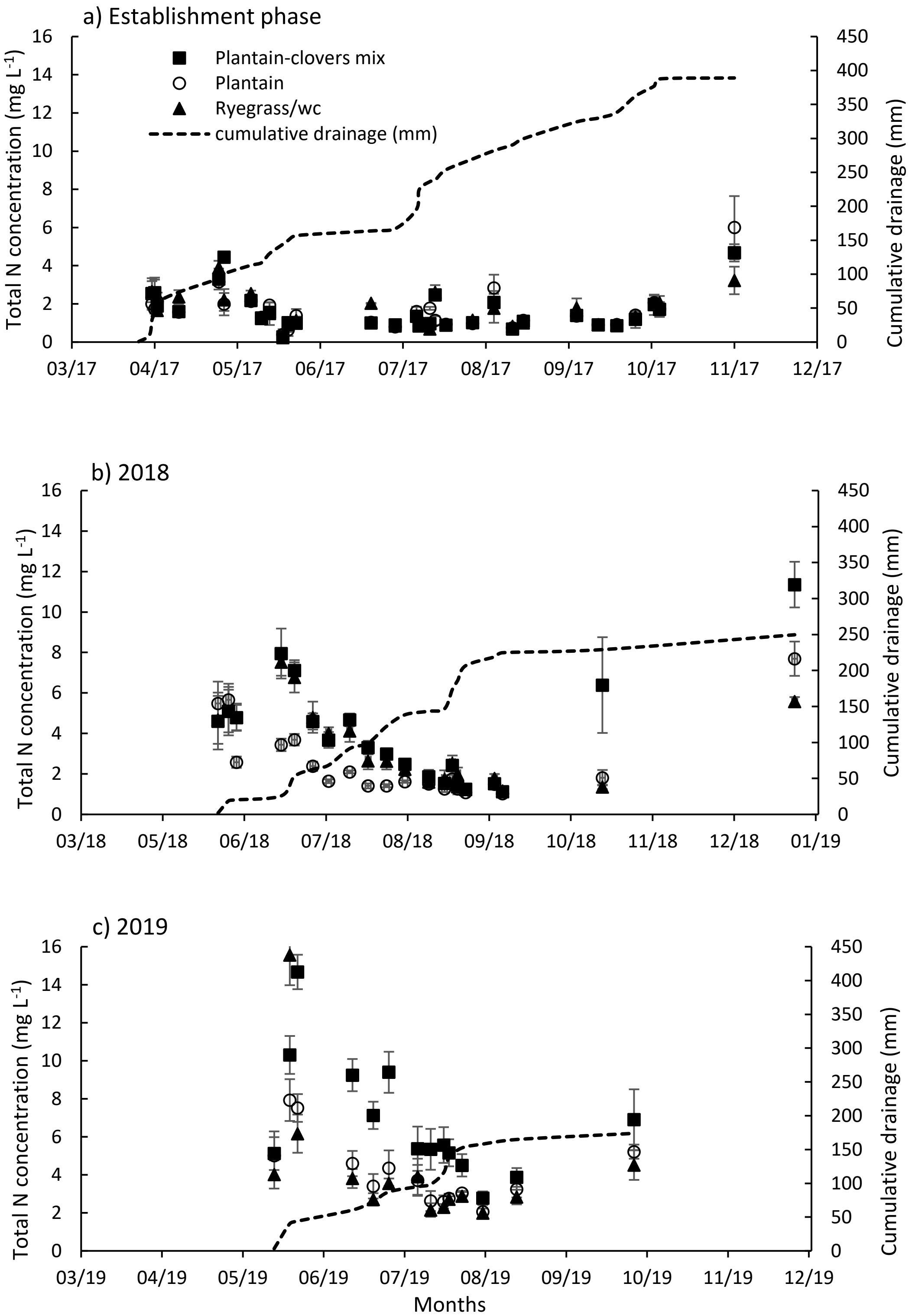


Figure 3.7. Total N concentration (mg N L^{-1}) in mole-pipe drainage water a)in the establishment phase, b) 2018 season and C) 2019 season, and the cumulative depths of drainage (mm) (dash line) for pure plantain (white circle), plantain-clovers mix (black square) and ryegrass-white clover (black triangle) pastures. Error bars represent the SEM at each sampling event (n=5).

Table 3.8. Total amount of nitrate-nitrogen (NO_3^-) and total nitrogen (TN) (kg N ha^{-1}) leached in drainage water during the establishment phase, 2018 and 2019 drainage seasons for plantain, plantain-clovers mix (Mix) and ryegrass-white clover (Ryegrass/wc) treatments, and the % of reduction in N leaching compared to ryegrass-white clover pasture. (Values are Mean ± SEM).

			N leached (kg N ha^{-1}year^{-1})	**% reduction in leaching compared to ryegrass-white clover**
Establishment phase 2017	NO_3^-N	Plantain	0.2 ± 0.03^{a}	87
		Plantain- clovers mix	0.2 ± 0.9^{a}	87
		Ryegrass/wc	1.2 ± 0.5^{b}	-
		***P* value**	0.02	
	TN	Plantain	1.3 ± 0.1^{a}	56
		Plantain- clovers mix	1.7 ± 0.2^{a}	45
		Ryegrass/wc	3.1 ± 0.1^{b}	-
		***P* value**	0.04	
2018	NO_3^-N	Plantain	2.8 ± 0.4^{a}	48
		Plantain- clovers mix	6.6 ± 0.4^{b}	-
		Ryegrass/wc	5.4 ± 0.5^{b}	-
		***P* value**	0.002	
	TN	Plantain	6.8 ± 0.2^{a}	21
		Plantain- clovers mix	9.4 ± 0.5^{b}	No reduction
		Ryegrass/wc	8.7 ± 1.0^{ab}	-
		***P* value**	0.03	
2019	NO_3^-N	Plantain	4.0 ± 0.4^{a}	No reduction
		Plantain- clovers mix	9.4 ± 1.2^{b}	No reduction
		Ryegrass/wc	3.1 ± 0.5^{a}	-
		***P* value**	0.0003	
	TN	Plantain	5.5 ± 0.4^{a}	19
		Plantain- clovers mix	11.6 ± 1.4^{b}	No reduction
		Ryegrass/wc	6.8 ± 0.8^{a}	-
		***P* value**	0.002	

Table 3.9. Soil mineral nitrogen (N), ammonium (NH_4^+-N) and nitrate (NO_3 N) (mg kg$^{–1}$ soil) in August 2018 and September 2019 from the experimental pastures. (Values are Mean ± SEM).

		NH_4^+-N (mg kg$^{–1}$ soil)		**NO_3^- -N (mg kg$^{–1}$ soil)**	
		0-7.5 cm	**7.5-15 cm**	**0-7.5 cm**	**7.5-15 cm**
August 2018	Plantain	10.2 ± 1.2	n.d.	2.5 ± 0.6	n.d.
	Plantain-clovers mix	10.6 ± 0.6	n.d.	5.9 ± 1.1	n.d.
	Ryegrass-white clover	13.5 ± 3.5	n.d.	3.9 ± 1.6	n.d.
	***P* value**	0.19		0.52	
September 2019	Plantain	3.6 ±1.4	0.9 ± 0.5	18.4 ± 1.0	17.9 ± 1.3
	Plantain-clovers mix	2.5 ± 1.2	1.4 ± 0.71	19.0 ± 1.5	18.9 ± 1.1
	Ryegrass-white clover	1.4 ± 0.8	0.4 ± 0.3	16.7 ± 0.8	23.1 ± 6.0
	***P* value**	0.39	0.40	0.37	0.43

n.d: not determined

3.5 DISCUSSION

During the establishment phase, the NO_3^- leaching losses for the three pasture treatments were very low. One explanation for these low losses is that only two grazing events occurred in late summer and autumn 2017, which is the critical period for the accumulation of NO_3^- in the soil that contributes to leaching during the next drainage season (Christensen *et al*., 2018). The Tokomau soil is a heavy soil and the denitrification rate could be high, therefore less NO_3^- could be accumulated in the soil during that period. The NO_3^- leaching losses mainly come from urine patches (Di & Cameron, 2002) and with less grazing events than would be usual in the summer and autumn period, then there would have been fewer patches to contribute to subsequent leaching losses.

In the 2018 drainage season, NO_3^- leaching losses from plantain pasture were lower than for ryegrass-white clover and plantain-clovers mix pasture treatments. Despite the similar total apparent N intake between cows fed plantain and ryegrass-white clover pastures, the NO_3^- leaching from plantain pasture was 48% (2.6 kg NO_3^--N ha^{-1}) lower compared to ryegrass-white clover pasture. In addition, the total N intake of cows fed plantain-clovers mix was on average 30% higher than ryegrass-white clover, and much greater during the critical period (late summer/ autumn), however, the NO_3^- leaching for both swards were similar. In early summer 2018, the apparent N intake was greater in cows fed plantain-clovers mix pastures than plantain and ryegrass-white clover pastures; and in autumn 2018, the apparent N intake by cows fed on the three pasture treatments was similar. Urine-N concentration from cows fed plantain and plantain-clovers mix pastures during the critical period was lower than ryegrass-white clover pastures despite the N intake from plantain-clovers being 50% greater than ryegrass-white clover over the critical period. During summer and autumn, in both pure plantain and plantain-clovers mix pastures the content of plantain in each pasture was greater than 30%. These results suggest that when plantain is in the diet, an effect is occurring on the animal which could be that either a lower proportion of the ingested N is partitioned to urine or a dilution effect on the urine (associated with a diuretic effect), which could explain the lower N leaching losses for the plantain pasture treatment, compared to the ryegrass-white clover pasture, even though N intakes were similar. Therefore, these results seem to support the contention that higher cow N intakes do not necessarily result in greater leaching when plantain is part of the pasture mix.

In the 2019 drainage season, the NO_3^- leaching losses between the plantain and the ryegrass-white clover pasture treatments were similar, and these losses were lower than NO_3^- losses from plantain-clovers mix pastures. The total apparent N intake by cows grazing plantain and ryegrass-white clover pastures were similar and these were lower than the N intake by cows fed plantain-clovers mix pastures. During the critical period, from late summer to autumn 2019, the apparent N intake was similar among the three pasture treatments, however, the UNc and urea-N concentrations in urine from cows fed plantain pastures was lower than the urine from cows fed ryegrass-white clover and plantain-clovers mix pastures, suggesting an effect of plantain in cows grazing only pure plantain pastures. The lack of effect of plantain on UNc and urea-N concentrations in urine of cows fed plantain-clovers mix suggests that as plantain comprised less than 20% of the sward there was insufficient plantain to significantly modify the N concentration in the urine (Minnée *et al.*, 2020). There may also have been an effect of the high clover content (ranging 22 to 42%) which could have increased the amount of N_2 fixed by the clover (Ledgard, 2001).

Dilution of the N excreted in the urine of cows fed plantain by an increase in the urine volume has been proposed as a mechanism that reduces the UNc (Box *et al.*, 2017; Mangwe *et al.*, 2019; Minnée *et al.*, 2020). Minnée *et al*. (2020) found greater urine volume when cows were fed 30% of plantain or more. The increase in urine volume results in an increase in the frequency of the urination rather than larger urination events (Mangwe *et al.*, 2019; Minnée *et al*., 2020). Some studies showed that when plantain was included in a diverse mix pasture (< 20%), there was no difference in the total urine volume and N excreted in the urine (Bryant *et al*., 2018; Edwards *et al*., 2015). The lack of a plantain effect on UNc was attributed to the similar apparent N intake for the two pasture treatments and that the proportion of plantain in the mix was lower than 20% (Bryant *et al.* 2018). In the current experiment, the reduction in UNc and urea-N concentrations in urine of cows fed with a pasture containing plantain was observed when plantain comprised more than 30% of the diet of the cows. In the 2018 drainage season, we can conclude that there was a dilution effect in the urine of cows fed pastures containing plantain which may decrease the urine patch N loading rate, decreasing N leaching from the system. In 2019, no effect of plantain was observed presumably because plantain made up less than 20% of the sward. During this season, the clover content in pure plantain increased and ranged between 9 and 17% of the DM, which could increase the N in the system (Ledgard, 2001). Therefore, this could reduce the beneficial effect of plantain on NO_3^- leaching.

The presence of bioactive compounds in plantain leaves (Stewart, 1996; Tamura & Nishibe, 2002) could also affect the UNc and urea-N concentrations in urine of cows grazing plantain. Plantain produces bioactive compounds with the potential to affect rumen fermentation and rumen N efficiency (Stewart, 1996). Navarrete *et al.* (2016) observed that aucubin had an inhibitory effect on rumen fermentation, reducing the rumen ammonia production. A reduction in ammonia formation could potentially reduce the urea-N concentration in the urine. In the present study, aucubin concentrations in plantain leaves were in the range of the values reported by Navarrete *et al.* (2016) (0.4 to 7.0 mg g^{-1} DM). The reduction in UNc and urea-N concentrations observed in urine of cows fed plantain in late summer could be related to high aucubin concentrations in plantain leaves. In autumn 2018, the aucubin concentration was low but the reduction in UNc and urea-N concentrations in urine was still significant in cows fed plantain pastures.

Greater partitioning of N to the faeces instead of urine could also explain the lower UNc and urea-N concentrations in the urine of cows grazing plantain, compared to ryegrass-white clover (Minnée *et al.* 2020; Totty *et al.* 2013). Minnée *et al.* (2020) observed that including 30% or more of plantain in the cows’ diets resulted in more N being partitioned to the faeces. This result was obtained after full collection of excreta from cows. Plantain traits that could be influencing the N partitioning in the cows are a reduction in soluble N and degradable N which could reduce the production of rumen NH_3 concentration, increasing the N partitioning to faeces which is less prone to be leached (Bryant *et al.*, 2019; Minnée *et al.* 2019). Plantain has also shown to have greater concentration of non-structural carbohydrates (NSC) compared with ryegrass. Therefore, the NSC/N ratio of the cows’diet has an effect on the N partitioning, reducing the proportion of dietary N eaten partitioned to urine (Bryant *et al.*, 2019; Minnée *et al.* 2019). However, Minnée *et al.* (2017) did not report any differences in faecal N concentrations from faeces spot samples, when plantain comprised either 20% or 40% of the daily DMI. Therefore, the effect of plantain on the N partitioning to faeces is not consistent and needs further investigation to understand how cows excrete surplus N in the total diet when plantain is incorporated into the diet.

Previous studies have also reported reduction in N losses when urine was applied to a pasture containing plantain (Carlton *et al*., 2018; Gardiner *et al*., 2017; Luo *et al*., 2018; Simon *et al*., 2019; Woods *et al*., 2018). Plantain may potentially affect soil processes via biological nitrification inhibition (Carlton *et al*., 2018; Gardiner *et al*., 2017; Luo *et al*., 2018; Woods *et al*., 2018). It has been observed that the inclusion of plantain reduced the abundance of soil ammonia oxidiser bacteria (AOB) compared to a soil growing ryegrass-white clover (Carlton *et al*., 2018). The results of inhibiting the AOB, a group of bacteria which contribute to the nitrification process in high-N soil environment (Di *et al*., 2009), is a greater soil NH_4^+ concentration and lower soil NO_3^- concentration, reducing the risk of NO_3^- leaching. The results from Carlton *et al*. (2018) indicate a reduction in the nitrification rate, decreasing the NO_3^- leaching losses under a plantain sward on average by 78% compared to ryegrass- white clover swards. This reduction in NO_3^- leaching has been attributed to the presence of bioactive compounds excreted by plantain roots (Dietz *et al.*, 2013; Gardiner *et al.*, 2017; Stewart, 1996; Tamura & Nishibe, 2002). While it would appear that plantain affected cow urine in the current study, little can be said about the effect of plantain on soil processes. The effect of plantain sward on N losses from the soil is assessed in the next two chapters of this thesis.

A number of studies have shown that the incorporation of plantain to the cows' diet does not reduce milk production (milk solids and milk yield) (Box *et al*., 2016; Dodd *et al.*, 2018; Minnée *et al.*, 2020; Totty *et al.*, 2013; Woodward *et al.*, 2012). In the current study, only in early spring 2017 did cows fed on plantain produce less milk solids than the plantain-clovers mix and the ryegrass-white clover pastures. The milk volume was similar between the three pasture treatments. During early/late summer 2019, cows fed on both plantain and plantain-clovers mix pastures produced a higher milk volume than cows grazing ryegrass-white clover pastures, however, the milk solids production was similar for the three pasture treatments in the 2018/2019 grazing season. Therefore, the environmental benefits of incorporating plantain to the cows' diets was clear in the 2017/2018 grazing season where NO_3^- leaching losses were reduced and the milk production was maintained.

In the 2018/2019 grazing season, although the milk solids production was similar among the three pasture treatments for each season, the pasture accumulated in plantain sward was lower (P= 0.05) than in ryegrass-white clover and plantain-clovers mix swards. This could be attributed to the low growth of plantain in winter and to the low persistence of plantain swards in this type of soil. According to Stewart (1996), although plantain can grow well on a range of soil conditions, it is not as tolerant as perennial ryegrass to treading effect or soil compaction. The soil in the current experiment is a poorly drained soil which could affect the growth and persistence of pure plantain during the second grazing season.

The reduction in NO_3^- leaching under plantain sward observed in the 2017/2018 grazing season was attributed to a dilution effect and possibly the effect of aucubin on the cow. According to Mangwe *et al*. (2019), total daily urine volume was more related to urination frequency than urine volume per event. Mangwe *et al*. (2019) reported that cows grazing plantain urinated 21.2 times per day, and those grazing ryegrass-white clover urinated 13.9 times/ day which was significantly less than that of cows grazing plantain. An increase in urination frequency by cows grazing plantain may increase the area covered by urine patches and potentially increase NO_3^- leaching and the cumulative N_2O emissions due to an increase in urine patches at the paddock scale. An increase in the area covered by urine patches could increase the mineralisation rate in those areas, affecting the amount of N loss as NO_3^- leaching and N_2O emissions. However, if the total amount of urine N excreted by cows grazing plantain does not increase, the N loss from the urine of cows grazing plantain will not increase (de Klein *et al*., 2019). Therefore, the effect of plantain sward on N_2O emissions and the plantain sward features that could affect the N_2O emissions from a plantain swards need further research.

3.6 CONCLUSIONS

Plantain reduced NO_3^- leaching losses compared to ryegrass-white clover pastures during the 2018 drainage season. The total N intake by cows grazing plantain was similar to ryegrass-white clover and these were lower than the N intake by plantain-clovers mix pastures. The reductions of UNc and urea-N concentrations in urine from cows fed plantain pastures were achieved during the critical period (summer to early winter), therefore, less NO_3^- is likely to have accumulated in the soil profile and NO_3^- leaching during the subsequent drainage events was smaller. Those reductions were due to a dilution effect by plantain sward on cow urine. In the 2019 drainage season, no difference in NO_3^- leaching losses were found between plantain and ryegrass-white clover pastures, which were both lower than NO_3^- leaching by plantain-clovers mix pastures. It is suggested that plantain had no effect this year because it comprised less than the critical value of 30% of the plantain sward. However, in the plantain-clovers mix sward the percentage of plantain was higher than 30% and there was no effect on NO_3^- leaching. Therefore, other factors such as clover content are important to consider to obtain a beneficial effect of plantain in a mixed sward. High clover content could increase the N in the biological system, increasing NO_3^- leaching losses. Reducing cow urinary N by feeding cows with pastures containing at least 30% plantain and keeping the clover content low, possibly less than 20 %, is a mitigation option that NZ farmers can use to reduce the NO_3^- leaching from pastoral systems without a reduction in milk yield.

3.7 REFERENCES

Blakemore, L. C. Methods for chemical analysis of soils. (1987). *NZ Soil Bur Sci Rep.*, *80*,71–76.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2016). Milk production and urinary nitrogen excretion of dairy cows grazing perennial ryegrass- white clover and pure plantain pastures. *New Zealand Society of Animal Production, 76,* 18-21.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2017). Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research, 60*(4), 470-482.

Bryant, R. H., Snow, V. O., Shorten, P. R., & Welten, B. G. (2020) Can alternative forages substantially reduce N leaching? findings from a review and associated modelling. *New Zealand Journal of Agricultural Research, 63*:1, 3-28.

Bryant, R. H., Welten, B. G., Costall, D., Shorten, P. R. & Edwards, G. R. (2018). Milk yield and urinary-nitrogen excretion of dairy cows grazing forb pasture mixtures designed to reduce nitrogen leaching. *Livestock Science, 209*, 46-53.

Cameron, K. C., Di, H. J. & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology, 162*(2), 145-173.

Carlsson, G. & Huss-Danell, K. (2003). Nitrogen fixation in perennial forage legumes in the field. *Plant and Soil, 253*(2), 353-372.

Carlton, A. J., Cameron, K. C., Di, H. J., Edwards, G. R. & Clough, T. J. (2018). Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. *New Zealand Journal of Agricultural Research*, *62,* 150-172.

Cheng, G., Mccormick, J., Hussein, A., Logan, C., Pacheco, D., Hodge, M. & Edwards, G. (2017). Live weight gain, urinary nitrogen excretion and urination behaviour of dairy heifers grazing pasture, chicory and plantain. *The Journal of Agricultural Science, 155*(4), 669-678.

Christensen, C., Hedley, M. J., Hanly, J. A. & Horne, D. J. (2018). Duration-controlled grazing of dairy cows. 2: nitrogen losses in sub-surface drainage water and surface runoff. *New Zealand Journal of Agricultural Research*: *62*(1), 48-68.

de Klein, C. A. M., van der Weerden, T. J., Luo, J., Cameron, K. C. & Di, H. J. (2019). A review of plant options for mitigating nitrous oxide emissions from pasture-based systems. *New Zealand Journal of Agricultural Research, 63*(1), 29-43.

Di, H. J. & Cameron, K. C. (2002). Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems, 64*(3), 237-256.

Di, H. J., Cameron, K. C., Shen, J. P. l., Winefield, C. S., O'callaghan, M., Bowatte, S. & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience, 2*(9), 621-624.

Dietz, M., Machill, S., Hoffmann, H. C. & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil, 368*(1-2), 445-458.

Dodd, M., Dalley, D., Wims, C., Elliott, D. & Griffin, A. (2018). A comparison of temperate pasture species mixtures selected to increase dairy cow production and reduce urinary nitrogen excretion. *New Zealand Journal of Agricultural Research*: *62*(4), 504-527.

Edwards, G., Bryant, R., Smith, N., Hague, H., Taylor, S., Ferris, A. & Farrell, L. (2015). Milk production and urination behaviour of dairy cows grazing diverse and simple pastures. *Proceedings of the New Zealand Society of Animal Production*, *75,* 79-83.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J., Edwards, G. R. & de Klein, C. A. M. (2017). Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. *New Zealand Journal of Agricultural Research*, *61* (4) 495-503.

Haynes, R. J. & Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in agronomy, 49,* 119-199.

Hewitt, A. E. (2010). New Zealand soil classification. *Landcare Research Science Series* (No.1), 136 pp.

Hodgson, J. (1979). Nomenclature and definitions in grazing studies. *Grass and Forage Science 34*(1),11-17.

Hosomi, M. & Sudo, R. (1986). Simultaneous determination of total nitrogen and total phosphorus in freshwater samples using persulfate digestion. *International Journal of Environmental Studies, 27*(3-4), 267-275.

Judson, H. G. & Edwards, G. R. (2016). Urinary nitrogen concentration from dairy heifers grazing kale supplemented with either plantain or perennial ryegrass baleage in winter. *Journal of New Zealand Grasslands, 78,* 99-102.

Leip, A., Billen, G., Garnier, J., Grizzetti, B., Lassaletta, L., Reis, S., Simpson, D., Sutton, M. A., de Vries, W., Weiss, F. & Westhoek, H. (2015). Impacts of European livestock production: nitrogen, sulphur, phosphorus and greenhouse gas emissions, land-use, water eutrophication and biodiversity. *Environmental Research Letters, 10*(11), 115004.

Luo, J., Balvert, S. F., Wise, B., Welten, B., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. & Judge, A. (2018). Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. *Science of The Total Environment*, *610–611,* 1271-80.

Mangwe, M. C., Bryant, R. H., Beck, M. R., Beale, N., Bunt, C. & Gregorini, P. (2019). Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. *Animal Feed Science and Technology, 252,* 11-22.

Minnée, E. M. K., Kuhn-Sherlock, B., Pinxterhuis, J. B. and Chapman, D. F. (2019) "Meta-analyses comparing the nutritional composition of perennial ryegrass (Lolium perenne) and plantain (Plantago lanceolata) pastures. *Journal of New Zealand Grasslands 81*: 117-123.

Minnée, E. M. K., Leach, C. M. T. & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science*, *239*, 104093.

Minnée, E. M. K., Waghorn, G. C., Lee, J. M. & Clark, C. E. F. (2017). Including chicory or plantain in a perennial ryegrass/white clover-based diet of dairy cattle in late lactation: Feed intake, milk production and rumen digestion. *Animal Feed Science and Technology, 227,* 52-61.

Navarrete, S., Kemp, P. D., Pain, S. J. & Back, P. J. (2016). Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata* L.) and their effect on *in vitro* rumen fermentation. *Animal Feed Science and Technology*, *222,* 158-67.

Scotter, D. R., Clothier, B. E. & Turner, M. A. (1979). The soil water balance in a gragiaqualf and its effect on pasture growth in central New Zealand. *Soil Research, 17*(3), 455-465.

Selbie, D. R., Buckthought, L. E. & Shepherd, M. A. (2015). The Challenge of the Urine Patch for Managing Nitrogen in Grazed Pasture Systems. *Advances in agronomy, 129,* 229-292.

Shepherd, M., Carlson, B. & Lucci, G. (2017). The effect of time of winter urine deposition on mineral nitrogen leaching and implications for grazed forage crops. *New Zealand Journal of Agricultural Research, 60*(4), 376-385.

Shepherd, M., Phillips, P. & Snow, V. (2011). The challenge of late summer urine patches in the Waikato region. In: Adding to the knowledge base for the nutrient manager. (Eds L.D. Currie and C L. Christensen). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 24. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. 8 pages.

Simon, P. L., de Klein, C. A. M., Worth, W., Rutherford, A. J. & Dieckow, J. (2019). The efficacy of Plantago lanceolata for mitigating nitrous oxide emissions from cattle urine patches. *Science of The Total Environment*, *691*, 430-441.

Stewart, A. V. (1996). Plantain (Plantago lanceolata) – a potential pasture species. *Proceedings of the New Zealand Grassland Association 58*,77-86.

Tamura, Y. & Nishibe, S. (2002). Changes in the Concentrations of Bioactive Compounds in Plantain Leaves. *Journal of Agricultural and Food Chemistry, 50*(9), 2514-2518.

Totty, V. K., Greenwood, S. L., Bryant, R. H. & Edwards, G. R. (2013). Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *Journal of Dairy Science, 96*(1), 141-149.

Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J. & Clough, T. J. (2018). Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. *Grass and Forage Science, 73*(4), 878-887.

Woodward, S.L., Waghorn, G.C., Bryant, M.A. & Benton, A. (2012). Can diverse pasture mixtures reduce nitrogen losses? In: Jacobs, J.L. (Ed.), Australasian Dairy Science Symposium. Department of Primary Industries, Melbourne, Australia, pp. 463–464.

World Health Organisation. (2011). Guidelines for drinking water quality. Geneva, Switzerland.

4 PLANTAIN DECREASES NITROGEN LOSSES TO THE ATMOSPHERE VIA A SWARD AND A URINE EFFECT

4.1 ABSTRACT

Urine patches deposited by grazing animals onto pastures are the main sources for nitrogen (N) losses to the atmosphere in New Zealand. Several studies have indicated that the use of novel swards such as plantain (*Plantago lanceolata* L.) can lead to reduced N losses from grazed systems to the environment. Two field experiments (spring 2017 and autumn/winter 2018) were conducted on a poorly drained Tokomaru silt loam soil to assess the effect of a plantain sward and urine from cows fed plantain sward on nitrous oxide (N_2O) and ammonia (NH_3) emissions compared to ryegrass (*Lolium perenne* L.)-white clover (*Trifolium repens* L.) swards. The N_2O emissions from soil were measured using the static chamber method, and NH_3 losses with the dynamic chamber method. In spring 2017, N_2O emissions from the plantain sward were lower ($P= 0.03$) than ryegrass-white clover sward. However, in autumn/winter 2018, the cumulative N_2O emissions and the emission factors from plantain swards were higher ($P< 0.0001$ and $P< 0.0001$, respectively) than ryegrass-white clover swards. This could be due to during this season it was observed that soil under plantain was wetter ($P< 0.05$) than ryegrass-white clover. In autumn/winter 2018, N_2O emissions from urine of cows grazing plantain was lower ($P= 0.003$) than urine from cows fed ryegrass-white clover swards. Urine from cows fed plantain swards reduced NH_3 losses compared to urine from the ryegrass-white clover treatment in both seasons. These results suggest that plantain sward could be a mitigation strategy for reducing N losses to the atmosphere from a dairy system, however, there are soil factors such as soil moisture content that could drive increasing N_2O losses from plantain swards in heavy soils.

Keywords: nitrous oxide, ammonia volatilisation, BNI (biological nitrification inhibition), *Plantago lanceolata* L., dairy farm

4.2 INTRODUCTION

In New Zealand, 95% of N_2O emissions come from agricultural sources, specifically from urine and dung patches produced by animals grazing all year round (Ministry for the Environment, 2019a). In these systems, animals usually consume more nitrogen (N) than needed for their physiological requirements such as those associated with growth. This excess N is excreted in urine and dung (Bolan *et al.*, 2004; Oenema *et al.*, 1997). Urine patches are small, localized volumes of soil with large quantities of available N. Nitrogen is urinated in patches at a rate equivalent to 200-1000 kg N ha^{-1} (Haynes & Williams, 1993). This amount of N exceeds the capacity of the plants to utilise it, increasing the risk to it being lost to the atmosphere as ammonia ($NH_{3(g)}$) volatilisation and nitrous oxide (N_2O) emissions. Fast urea hydrolysis starts within hours of urine deposition, resulting in high NH_3 losses (Bolan *et al.*, 2004; Petersen *et al.*, 1998; Ryden *et al.*, 1987) which may act as a secondary source of N_2O emissions (Saggar *et al.*, 2005). Ammonia volatilisation can cause eutrophication and acidification of water and soils where it is re-deposited (Misselbrook *et al.*, 2013). Loss of N as $NH_{3(g)}$ from urine patches ranges between 7% and 15% of the total N applied as urine (Rodriguez *et al.*, 2019; Zaman *et al.*, 2013).

Nitrous oxide is a greenhouse gas (GHG) with a global warming potential 298 times greater than carbon dioxide (CO_2) (IPCC, 2014), and it also contributes to stratospheric ozone depletion (Ravishankara *et al.*, 2009). Nitrous oxide is produced by the soil microbial processes of nitrification and denitrification, which have been well described in previous studies (Baggs & Philippot, 2010; Bremner, 1997; Saggar *et al.*, 2013). The Paris Agreement is a global agreement on climate change and all participant countries have committed to reduce their GHG emissions (Ministry for the Environment, 2019b). Under this agreement, New Zealand has committed to reduce its GHG emissions by 30% below 2005 levels by 2030. Recently the Government's Zero Carbon Bill aims to reduce all GHG, and N_2O to net zero by 2050 (Ministry for the Environment, 2019c).

Strategies to mitigate the environmental impacts of N_2O losses such as nitrification inhibitors, restricted winter grazing and diet manipulation have been developed in previous studies (Christensen *et al.*, 2018; Di & Cameron, 2002, 2016; Luo *et al.*, 2010; Misselbrook *et al.*, 2005). Recently in New Zealand, researchers have shown plantain (*Plantago lanceolata* L.) has the potential to reduce N_2O emissions. Plantain is a herb pasture plant that produces and may release secondary metabolites with potential biological nitrification inhibitor properties (de Klein *et al*., 2019; Gardiner *et al*., 2019; Luo *et al*., 2018; Pijlman *et al*., 2019; Simon *et al*., 2019). Luo *et al*. (2018) found that a plantain sward can reduce N_2O emissions from a urine patch by 74%. Simon *et al*. (2019) showed that increasing the proportion of plantain in perennial ryegrass-white clover (*Lolium perenne* L. and *Trifolium repens* L.) (ryegrass-white clover) pastures reduced N_2O emissions due to a decrease in urinary N concentration. Moreover, Simon *et al*. (2019) also found that when the urine of cows feeding on a standard ryegrass-white clover was applied to swards with an increasing proportion of plantain (0, 30, 60 and 100% plantain), N_2O emissions decreased linearly with an increasing percentage of plantain.

The use of plantain swards on farms could potentially mitigate losses of N from urine patches to the atmosphere. Studies under different environmental and soil conditions are needed to determine how these different conditions affect N_2O emissions from plantain swards and relative contribution of urine or soils. Therefore, the aim of this study was to evaluate the effects of plantain sward on N_2O and NH_3 losses from urine patches by applying urine from cows fed plantain and ryegrass-white clover to plantain and ryegrass-white clover swards. We hypothesised that: i) the plantain sward will reduce N_2O emissions from urine patches regardless of the type of urine; however, an increase in NH_3 volatilisation might be observed; and ii) N_2O emissions and NH_3 losses resulting from deposition of urine of cows grazing a plantain sward will be lower than the urine of cows fed a ryegrass-white clover sward.

4.3 MATERIALS AND METHODS

4.3.1 Site description

The research was conducted at Massey University’s Dairy Farm 4, near Palmerston North (40° 39’ S; 175 ° 61’ E). The soil in the paddock is Tokomaru silt loam, which is classified as an Argillic-fragic Perch-gley Pallic Soil (Hewitt, 2010). A detailed description of soil physical properties is provided by Scotter *et al.* (1979). This experiment was set up in the plantain and ryegrass-white clover adaptation paddocks, in the main field experiment described in Chapter 3. The experimental area was fenced off three months before the experiment to avoid excreta deposition from grazing cows and to minimise the effect of previous dung and urine patches (de Klein *et al.*, 2014).

4.3.2 Experimental design

Two field experiments were conducted in (i) spring (29th September- 8th November 2017) and (ii) in autumn/winter (4th May- 26th July 2018). The research area, in both experiments, comprised three areas where all treatments were applied at the same time. These three areas (Figure 4.1) were used for: (i) N_2O chambers, (ii) NH_3 volatilisation chambers and (iii) plots for soil sampling. The experiments were laid out in a 2 x 2 factorial design with two sward treatments and two urine treatments with 5 replicates each treatment. The sward treatments were: pure plantain, (cv. Tonic), and ryegrass-white clover (cv. Trojan and ‘Emerald’, respectively); and the two urine treatments were: urine of cows grazing either pure plantain, or ryegrass-white clover swards. A water treatment (control) was also applied to the sward treatments. All treatments are described in Table 4.1. The plantain and ryegrass-white clover swards were cut to 5 cm above ground level and plant material was removed from the plots before the application of the urine and water.

Urine was applied at a hydraulic loading rate of 10 L m^{-2} (equivalent to 10 mm application depth). According to Selbie *et al.* (2015), this hydraulic rate is typical for urine patches from dairy cows. In the N_2O chambers and soil plots, urine was applied using a metal ring (0.5 m diameter) that was removed after urine infiltrated into the soil. Control treatments received the same volume of water. For the NH_3 plots, urine was applied directly inside the chambers (0.19 m diameter).

In each soil plot (0.5 m x 0.5 m separated by a 0.5 m buffer), soil samples were taken, N_2O was sampled from the chambers and NH_3 was sampled from the acid trap. The sampled soil was analysed for ammonium (NH_4^+-N) and nitrate (NO_3^--N) and soil moisture content. Temperature inside the N_2O and NH_3 chambers, outside the chambers and the soil temperature were recorded during the experiment using logger thermometers.

Table 4.1. Treatments used in both field experiments.

Sward (S)	Urine source (U)	Treatment name
Ryegrass-white clover	Ryegrass-white clover	$S_{r/w}\_U_{r/w}$
	Plantain	$S_{r/w}\_U_{pl}$
	Water	$S_{r/w}$_Water
Plantain	Ryegrass-white clover	$S_{pl}\_U_{r/w}$
	Plantain	$S_{pl}\_U_{pl}$
	Water	S_{pl}_Water

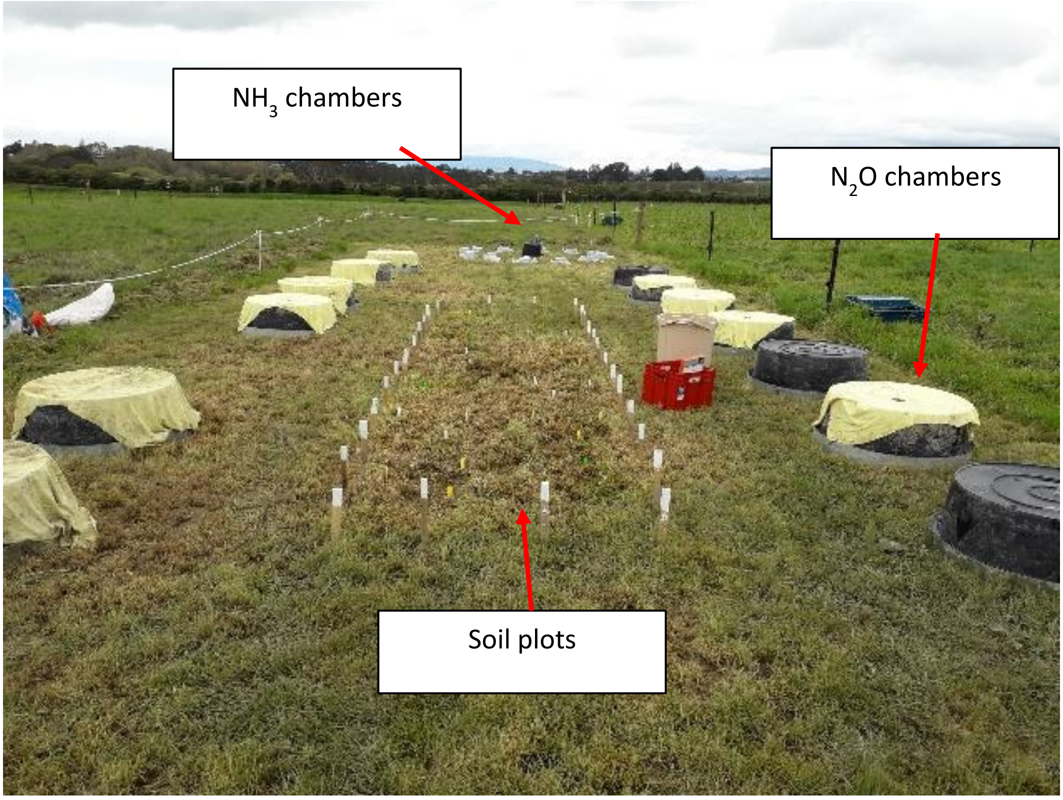


Figure 4.1. Layout of the experiment in ryegrass-white clover swards. Same layout was established in plantain swards.

4.3.3 Urine collection and analyses

Urine was collected from cows grazing a plantain sward and from cows grazing a ryegrass-white clover sward (cows grazing the plots in the main field experiment, during spring 2017 and autumn 2018, Chapter 3). Urine was collected after 7-8 days of grazing the treatment sward, immediately after the morning milking. After collection, the urine for each diet was bulked and stored at 4 °C until application to avoid urea hydrolysis, and it was applied 24 h after the collection. A sub-sample (100 mL) of each urine type was taken immediately after collection for analysis of total N (TN) and urea-N concentration.

Total N in urine samples was analysed by the Kjeldahl digestion method (McKenzie & Wallace, 1954). Urea-N was determined by the colorimetric method in which the red colour is formed when the extraction is heated along with diacetyl monoxime (DMA), thiosemicarbazide (TSC), sulphuric and orthophosphoric acid and ferric chloride hexahydrate (Douglas & Bremner, 1970; Mulvaney & Bremner, 1979).

4.3.4 Nitrous oxide sampling

After urine application in the spring 2017 experiment, N_2O fluxes were measured 9 times over a period of 60 days. In the autumn/winter 2018 experiment, N_2O fluxes were measured 19 times over a period of 84 days. Nitrous oxide samples were collected using the static chamber method. Each chamber had an 0.80 m internal diameter and a height of 0.30 m, and a volume of 151 L. Galvanised metal rings (0.80 m diameter), one for each chamber, were inserted 0.10 m into the soil; and remained in the field until the end of each experiment. On each sampling day the chambers were placed in the galvanised metal rings and sealed with bicycle tubes, which were inflated to provide a gas-tight seal (Appendix 1). Then the chamber was covered by a wet towel to help to maintain a constant temperature inside the chambers. Chambers were closed for two hours and, due to the large volume of the chambers, gas samples were taken at 0, 60 and 120 minutes. Time 0 started as soon as each chamber was closed. Gas samples (25 mL) were collected from a sampling port on the chamber using a plastic syringe and injected through a septum into evacuated sample vials. Nitrous oxide sample collections were carried out between 11:00 am and 2:00 pm on each sampling day, which according to van der Weerden *et al*. (2013) allows extrapolation to a daily flux without bias. In both experiments, the first 4 weeks N_2O samples were taken twice a week and for the remainder of the experiment, samplings were conducted once per week until background levels were reached. On each sampling day, three background air samples were also taken at each time.

After sampling, the vials analysed for N_2O concentration via gas chromatography using a Shimadzu Nexis 2030 gas chromatograph. The emissions over the sampling time were integrated over time for each chamber to estimate the total emissions during the measurement period (Luo *et al*., 2013).

The N_2O fluxes were calculated from the slope of the linear increase of N_2O concentration in the chamber over time (de Klein *et al*., 2003) using the following equation:

$$N_2O \text{ flux (mg N m}^{-2}\text{ h}^{-1}) = \frac{\delta N_2O}{\delta T} * \frac{M}{Vm} * \frac{V}{A} \qquad (4.1)$$

where δ N_2O is the increase in N_2O in the headspace (µL L^{-1}); δT is the enclosure period (hours); M is the molar weight of N in N_2O; Vm is the molar volume of the gas at the sampling temperature (L mol^{-1}); V is the headspace volume (m^3) and A is the area covered (m^2). The N_2O fluxes were converted to kg N_2O-N ha^{-1} day^{-1}.

Emissions, expressed as % reduction, from treatments were compared with those from the ryegrass-white clover sward with ryegrass-white clover urine application as it was assumed that this treatment would emit more N due to the greater N concentration in this urine. The total N_2O emission for each treatment over the experimental periods was calculated by integrating the emissions per day over time. The N_2O emission factors (EF_3) were calculated using the following equation:

$$EF_3 = \frac{N_2O\text{ -N total (urine) -}N_2O\text{-N total (control)}}{\text{Urine N applied}} \qquad (4.2)$$

where N_2O-N total (urine) and N_2O-N total (control) are the cumulative N_2O-N emissions for the measurement periods from the urine and control (water) chambers, respectively (kg N ha^{-1}), and Urine N applied is the rate of urine N applied (kg N ha^{-1}).

4.3.5 Ammonia volatilisation sampling

Ammonia volatilisation was measured using the dynamic chamber method (Kissel *et al.*, 1977) that comprised a volatilisation chamber and an acid trap to capture the NH_3. Chambers (0.19 m diameter, 0.04 m total height) of PVC with transparent lids (to allow photosynthesis) were inserted into the soil to a depth of 0.01 m, giving a headspace volume of 0.0011 m^3. Each chamber was connected to an acid trap, containing sulfuric acid (10 mL, 0.5 M H_2SO_4) using a tube connected to an aquarium pump. Air from the chambers was sucked at a constant flow rate (1 L min^{-1}) and passed through the acid trap. Sub-samples of the H_2SO_4 solution in the acid traps were analysed for NH_4^+-N concentrations, as below. Samples were taken every day for the first 7 days, and then on days 10, 13, 16 and 20.

4.3.6 Soil sampling and measurements

Before treatment application, soil samples were collected from the area of the experiment. Following the application of the treatments, soil samples (25 mm diameter and 450 mm deep; sectioned in 4 depths: 0-75, 75-150, 150-300 and 300-450 mm) were collected periodically from the soil plots adjacent to the N_2O and NH_3 chambers. Soil samples were taken 1, 7, 14, and 30 days after the treatment application, and again at the end of the experiment. At each sampling, three soil cores were bulked to produce a single sample of each soil plot. A sub-sample of 3 g of field moist soil was extracted with 25 mL of 2M potassium chloride (KCl). The extract was analysed for NO_3^--N and NH_4^+-N concentrations colorimetrically using a Technicon AutoAnalyser (Blakemore, 1987). A subsample of the wet soil was weighed and then oven dried at 105 ^{o}C for 24 hours. After 24 h drying, each sample was weighed again, and the gravimetric water content was calculated.

Water-filled pore space (WFPS %) was calculated using the gravimetric soil moisture content, bulk density and a particle density assumed to be 2.65 Mg m^{-3} (Luo *et al.*, 2018; Simon *et al.*, 2019). No difference in WFPS between plantain and ryegrass-white clover swards were observed. During these experiments, samples for moisture content were taken from 0-75 mm. Therefore, in autumn 2019 a small field experiment was conducted, which involved taking soil samples (25 mm diameter) from 0-50 mm depth under both swards. Sampling started when the soil was still relatively dry and continued until July. Soil samples were taken before a rainfall event and two days after it to allow time for the soil to drain. At each sampling, seven soil cores were bulked to produce a single sample from each plot, resulting in five replicates per sward.

4.3.7 Pasture analysis

Pasture was cut at 5 cm height twice during the experimental periods (approximately day 30 and 60 after treatment applications), and the herbage was oven-dried at 65°C for 48 hours and weighed. The dried herbage was finely ground and analysed for total N concentration. Total N was determined by combustion using a Leco analyser (AOAC, 2020; method 968.06). The cumulative dry matter (DM) yield (DM ha^{-1}) was calculated for each chamber.

4.3.8 Statistical analysis

Data were analysed using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Cumulative N_2O and NH_3 losses, soil mineral N, total N and DM in the pastures were analysed as a 2 x 2 factorial randomised design with soil and urine as fixed effects and replicates as random effects. The normal distribution of the data were checked. If the data sets did not meet the assumption of normality, they were log transformed. The N_2O data were log transformed before analysis and back-transformed to present means.

Regression analysis was carried out in both field experiments between N_2O emitted in the first month and soil and pasture parameters.

4.4 RESULTS

4.4.1 Urine composition

In the spring 2017 experiment, TN concentrations in urine from cows fed plantain and ryegrass-white clover pastures were 2810 and 3180 mg L^{-1}, respectively (Table 4.2). Urea-N concentration was only 27% and 44% of the TN in urine from cows fed plantain and ryegrass-white clover treatments, respectively. The urine applied in the autumn/winter 2018 experiment had higher concentrations of TN and urea-N than in spring. Total N concentration in urine from cows fed plantain and ryegrass-white clover pastures were 3850 and 4990 mg L^{-1}, respectively. In the urine of cows fed plantain treatment, 59% of the total N was as urea-N and in the urine from cows fed ryegrass-white clover, urea-N represented 74% of the TN.

Table 4.2. Total nitrogen (N), urea-N and percentage of the urea-N reduction of the dairy cow urine used in both spring 2017 and autumn/winter 2018 experiments (n=3).

	Urine	**Total N (mg L^{-1})**	**Urea-N (mg L^{-1})**	**% of urea-N reduction***
Spring 2017	Plantain	2810	768 (27% of TN)	45
				-
	Ryegrass-white clover	3180	1397 (44% of TN)	
Autumn/winter 2018	Plantain	3850	2243 (59% of TN)	40
	Ryegrass-white clover	4990	3710 (74% of TN)	-

* Percentage of urea-N concentration reduction in plantain urine compared to ryegrass-white clover urine for each season.

4.4.2 Weather and soil moisture conditions

During the spring 2017 experiment (28th September to 4th November 2017), the total rainfall was 97 mm and this period was characterised by a decline in the soil moisture content (Figure 4.2a). The soil water deficit (SWD) during the first 15 days of the experiment was nearly zero; subsequently, a period of low rainfall commenced and by the beginning of November a SWD of about 25 mm had developed (Figure 4.2a). Generally, the plantain soil showed greater soil moisture contents than the ryegrass-white clover soil, but it was not significantly different. During the spring experiment, the daily temperature ranged from 3 °C to 25 °C. Soil WFPS values ranged from 22 to 78% under the plantain sward and from 25 to 71% in the ryegrass-white clover sward during the experimental period (Figure 4.3a).

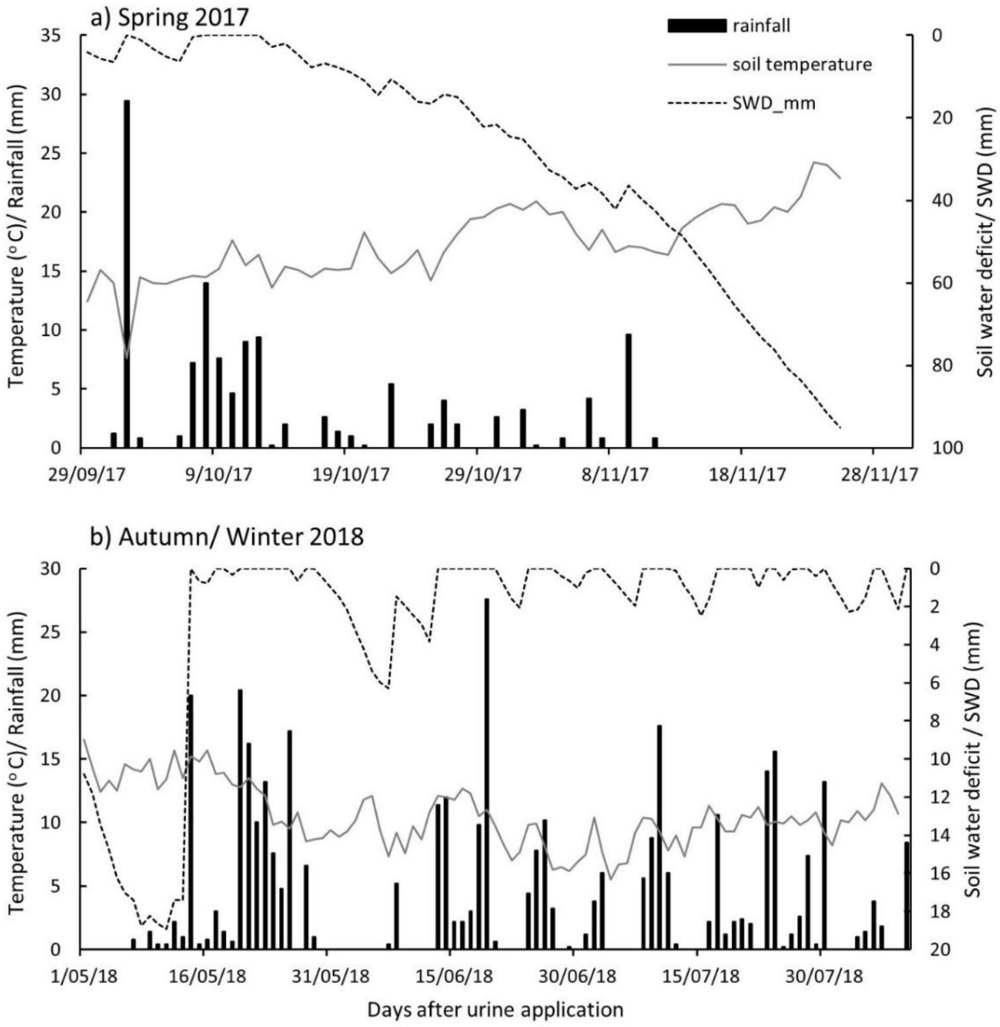


Figure 4.2. Daily rainfall (black bars), mean daily soil temperature (°C) at 10 cm depth (grey line) and soil water deficit (SWD) (mm) (dash line) in (a) spring 2017 and (b) autumn/winter 2018.

During the autumn/winter 2018 experiment (4th May to 26th July 2018) the soil had a high moisture content due to high rainfall (313 mm) (Figure 4.2b). During the first 10 days of the experiment, the SWD was between 15-19 mm, however, subsequently it was at or near field capacity. Soil WFPS values ranged from 47 to 80% (Figure 4.3b). Water filled pore space under the plantain sward were similar to ryegrass-white clover for the first part of the experiment, but greater than ryegrass-white clover at the end of the experiment ($P< 0.05$). During this experiment, the average daily temperature ranged from 0 °C to 22 °C.

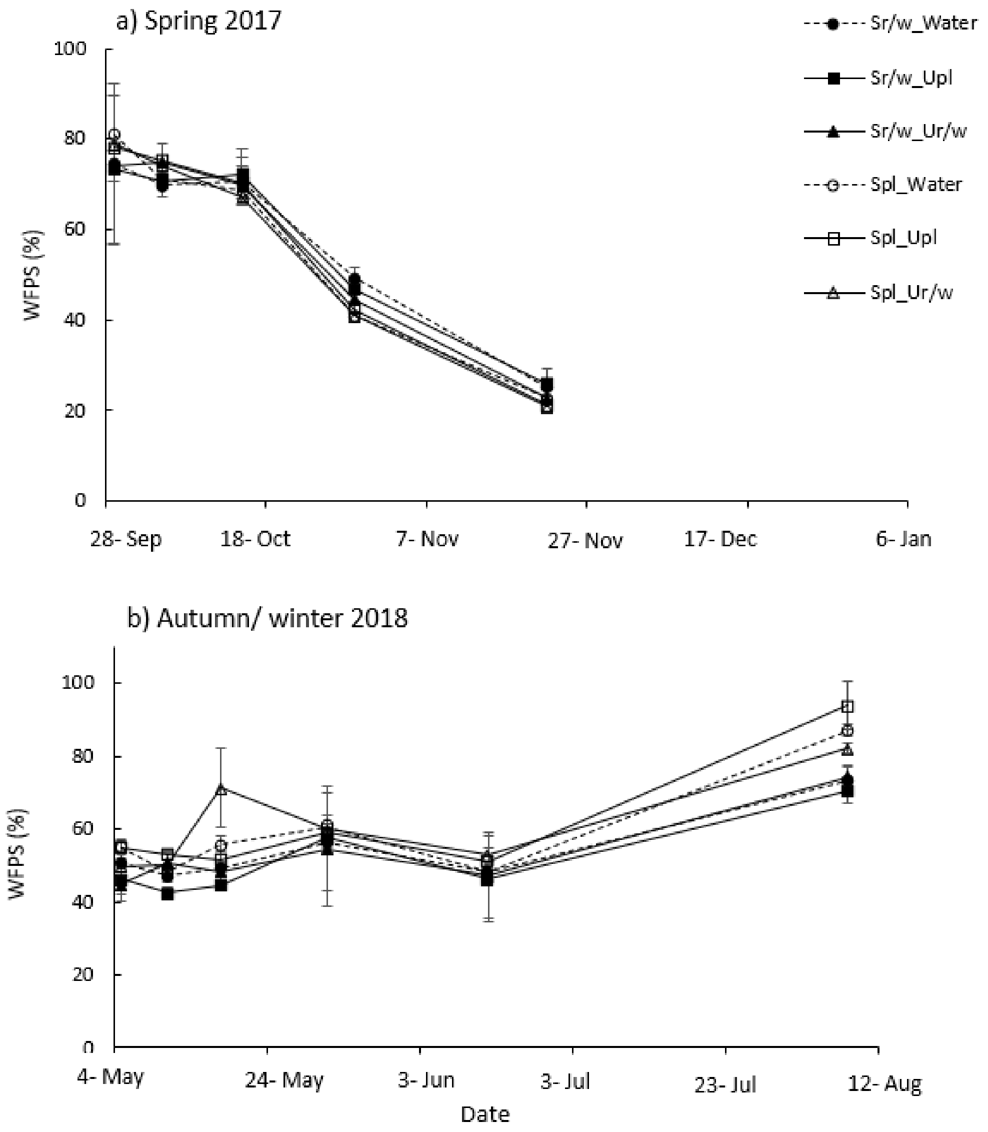


Figure 4.3. Soil water filled-pore space (WFPS %) in pure plantain and ryegrass-white clover swards, treated with urine from cows fed pure plantain and ryegrass-white clover swards; a control was also included where water was applied. (a) Spring 2017 experiment; (b) Autumn/winter 2018 experiment. Error bars are standard error of the mean (SEM) (n=4). $S_{r/w}$_= ryegrass-white clover sward, $U_{r/w}$ = ryegrass-white clover urine; S_{pl}= plantain sward; U_{pl} = plantain urine.

Figure 4.4 shows the WFPS from soil samples taken from late autumn through to late winter 2019. Water filled pore space was significantly higher (P= 0.05 and P = 0.04) in plantain than ryegrass-white clover on 8th July 2019 and 14th July 2019.

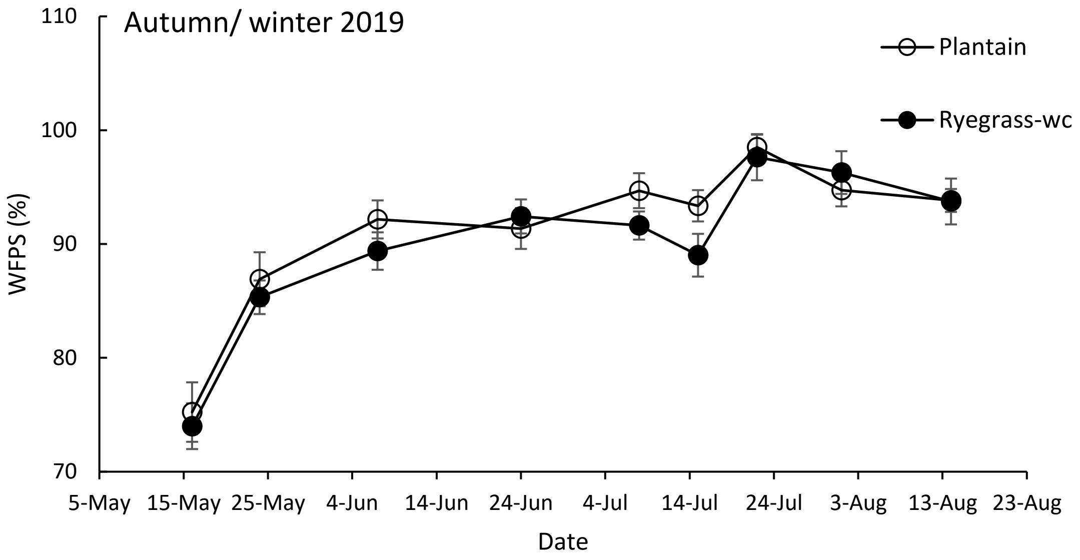


Figure 4.4. Soil water filled pore space (WFPS %) in plantain and ryegrass-white clover swards in autumn/winter 2019. Error bars indicate the SEM (n=5).

4.4.3 Nitrous oxide emissions

Daily N_2O fluxes showed temporal variations and the emissions from all treatments sharply increased after urine application in both spring and autumn/winter experiments. In both experiments, N_2O fluxes varied in response to rainfall events. At the beginning of the spring experiment, heavy rainfall events occurred immediately after urine application and measurements had to be delayed 4 days due to the galvanised metal rings being flooded.

Nitrous oxide fluxes from the water control treatments in both experiments ranged between 0.001 and 0.161 kg N-N_2O ha^{-1} d^{-1}, remaining constant and low during both experimental periods.

During the spring 2017 experiment, all urine treatments exhibited only one N_2O peak on day 22 after urine application regardless of the sward treatment (Figure 4.5a). On that day, the highest N_2O peak was from $S_{r/w}\_U_{r/w}$ (0.37 kg N_2O-N ha^{-1} day^{-1}). For both urines, the cumulative N_2O emissions from the ryegrass-white clover sward were significantly higher (*P* = 0.03) than from the plantain sward (Table 4.3). However, there was no effect of the urine treatment on N_2O emissions (*P*= 0.27). Emissions factors (EF_3) in spring were similar (*P*= 0.79) between the ryegrass-white clover and plantain swards (Table 4.3).

During the autumn/winter 2018 experiment, in both the ryegrass-white clover and plantain swards, N_2O emissions exhibited major peaks on three days, 11, 19 and 43 after urine application (Figure 4.5b). After day 57, N_2O emissions reached the background level. Contrary to the results from spring 2017, in autumn/winter 2018, for both urines, the cumulative N_2O emissions from the plantain sward (10.3 kg N_2O-N ha^{-1}) were significantly higher (*P*< 0.0001) than from ryegrass-white clover (3.2 kg N_2O-N ha^{-1}) sward, with the calculated EF_3 values for this period being significantly higher (*P*< 0.0001) for the plantain sward. Cumulative N_2O emissions from plantain urine (4.6 kg N_2O-N ha^{-1}) were lower (*P*= 0.003) than emissions from ryegrass-white clover urine (8.9 kg N_2O-N ha^{-1}). The EF_3 were higher (*P*= 0.03) for ryegrass-white clover urine than plantain urine on both the plantain and ryegrass-white clover swards (Table 4.3).

During this experiment, there were no strong relationships between cumulative N_2O emitted and WFPS, soil mineral N or N uptake by pastures.

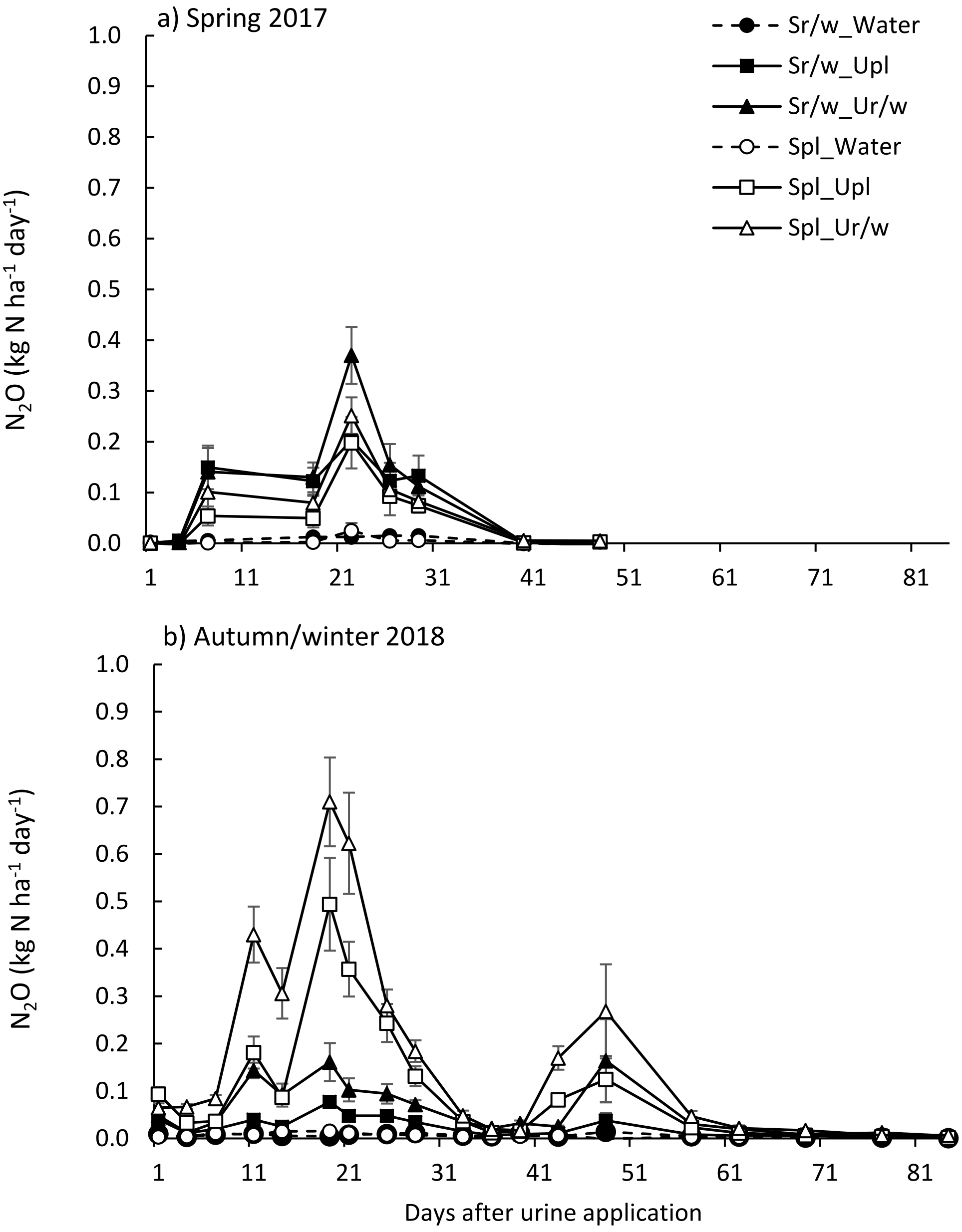


Figure 4.5. Nitrous oxide fluxes (kg N_2O-N ha^{-1} day^{-1}) from urine patches in pure plantain and ryegrass-white clover swards, treated with urine from cows fed pure plantain and ryegrass-white clover pastures; a control was also included were water was applied. (a) Spring 2017 experiment; (b) Autumn/winter experiment 2018. Error bars are SEM (n=5). $S_{r/w}$= ryegrass-white clover sward, $U_{r/w}$= ryegrass-white clover urine; S_{pl}= plantain sward; U_{pl} = plantain urine.

During the first month of the autumn/winter 2018 experiment, there were significant linear relationships between N_2O losses and WFPS (P= 0.01) were found. Pairwise relationships between cumulative N_2O and DM were weak.

Table 4.3. Cumulative N_2O emissions (kg N-N_2O ha^{-1}), percentage of change in N_2O emissions and emission factors (EF_3) for both spring 2017 and autumn/winter 2018, from plantain and ryegrass-white clover swards when plantain and ryegrass-white clover urine were applied. (Values are mean ± SEM).

	Spring 2017			**Autumn/Winter 2018**		
	Cumulative N_2O (kg N ha^{-1})	Change in N_2O (%)*	EF_3 **	Cumulative N_2O (kg N ha^{-1})	Change in N_2O (%)*	EF_3 **
$S_{r/w}$_$U_{r/w}$	4.8 ±0.82	n.a.	1.4 ±0.29	4.7 ±1.03	n.a.	0.8 ±0.21
$S_{r/w}$_U_{pl}	4.2 ±0.60	-13.8	1.4 ±0.21	1.7 ±0.23	-63	0.3 ±0.06
$S_{r/w}$_Water	0.4 ±0.02	n.a.	n.a.	0.5 ± 0.13	n.a.	n.a.
S_{pl}_$U_{r/w}$	3.5 ±0.48	-28	1.0 ±0.19	13.1 ±1.99	+177	2.5 ±0.40
S_{pl}_U_{pl}	2.8 ±0.56	-42	0.9 ±0.17	7.4 ±0.92	+157	1.8 ±0.24
S_{pl}_Water	0.2 ±0.08	n.a.	n.a.	0.5 ±0.10	n.a.	n.a.
Significance						
Sward type	0.03		0.79	<0.0001		<0.0001
Urine type	0.27		0.69	0.003		0.03
Interaction	0.85		0.88	0.29		0.71

*Change in N_2O (%) in comparison with ryegrass-white clover urine applied to ryegrass-white clover sward for each season; n.a. not applicable; **EF_3: emission factor of the applied urine N emitted as N_2O.

4.4.4 Ammonia volatilisation

In both experiments, large NH_3 losses were observed in those treatments where urine was applied compared to control treatments (Table 4.4). In the spring 2017 and in the autumn/winter 2018 experiments, cumulative NH_3 losses from the plantain and ryegrass-white clover swards were not different (P= 0.20 and P= 0.28, respectively) (Table 4.4). In both seasons, total NH_3 losses from plantain urine treatments were significantly lower (P= 0.002 and P< 0.0001, respectively) compared to NH_3 losses from ryegrass-white clover urine (Table 4.4).

In both experiments, NH_3 emissions for the water control treatments were very low and ranged from 0.001 to 0.01 kg NH_3-N ha^{-1} during the experimental periods.

Table 4.4. Cumulative NH_3 (kg N-NH_3 ha^{-1}) losses and percentage of reduction in NH_3 losses compared to $S_{r/w}$_$U_{r/w}$ treatment for both spring 2017 and autumn/winter 2018, from plantain and ryegrass-white clover swards when plantain and ryegrass-white clover urine were applied. (Values are mean ± SEM).

	Spring 2017		**Autumn/winter 2018**	
Treatments	Cumulative NH_3 (kg N-NH_3 ha^{-1})	Change of NH_3 compared to $S_{r/w}$_$U_{r/w}$(%)	Cumulative NH_3 (kg N-NH_3 ha^{-1})	Change of NH_3 compared to $S_{r/w}$_$U_{r/w}$(%)
$S_{r/w}$_$U_{r/w}$	15.7 ± 2.65	n.a.	6.7 ± 1.83	n.a.
$S_{r/w}$_U_{pl}	2.9 ± 0.35	-82	0.9 ± 0.15	-86
$S_{r/w}$_Water	0.04 ± 0.01	n.a.	0.2 ± 0.09	n.a.
S_{pl}_$U_{r/w}$	9.7 ± 3.42	-38	9.7 ± 1.61	-46
S_{pl}_U_{pl}	2.2 ± 0.74	-86	0.7 ± 0.05	-90
S_{pl}_Water	0.02 ± 0.01	n.a.	0.1 ± 0.03	n.a.
Significance				
Sward type	0.20		0.28	
Urine type	0.002		<.0001	
Interaction	0.30		0.20	

n.a. not applicable.

4.4.5 Soil mineral N

In spring 2017, soil ammonium peaked at 7 days after urine application, reaching 345 mg NH_4^+-N kg^{-1} in $S_{r/w}\_U_{r/w}$ treatment, followed by $S_{r/w}\_U_{pl}$, $S_{pl}\_U_{pl}$ and $S_{pl}\_U_{r/w}$ (260, 245 and 202 mg NH_4^+-N kg^{-1}, respectively). Following day 7 after urine application, soil ammonium in both swards gradually decreased reaching control treatment concentrations 31 days after urine application (Figure 4.6a). Soil NO_3^--N concentrations were initially low after urine application. Then, soil NO_3^--N increased over time peaking at 31 days after urine application in $S_{pl}\_U_{r/w}$, $S_{pl}\_U_{pl}$ and $S_{r/w}\_U_{r/w}$, except for $S_{r/w}\_U_{pl}$ which peaked after 18 days of urine application (Figure 4.6b).

In autumn/winter 2018, soil NH_4^+-N concentrations peaked on day 7 after urine application, with higher ($P < 0.05$) values for urine from cows fed ryegrass-white clover than plantain swards (Figure 4.6c). The NH_4^+-N concentrations gradually decreased until day 29 when they were similar to control treatments. Soil NO_3^--N concentrations increased gradually, peaking on day 14 in all treatments. Soil NO_3^--N from plantain swards on days 7, 14 and 30 were higher ($P= 0.05$) than ryegrass-white clover swards. Soil NO_3^--N reached background levels after day 51.

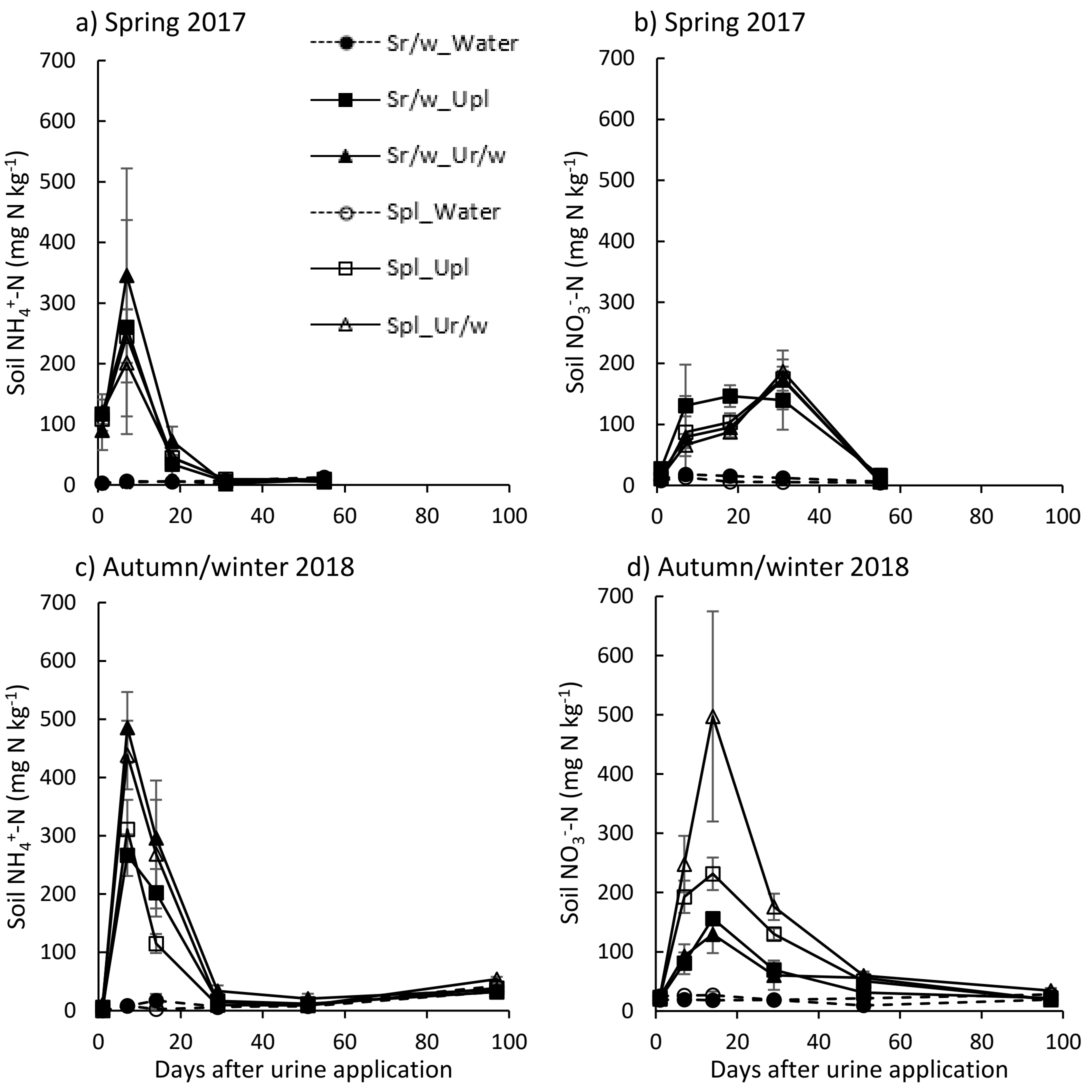


Figure 4.6. Soil ammonium (NH_4^+-N), and soil nitrate (NO_3^- -N) concentrations (mg N kg^{-1}) in spring 2017 (a and b) and in autumn/winter 2018 (c and d). Error bars are SEM (n=4). $S_{r/w}$= ryegrass-white clover sward, $U_{r/w}$ = ryegrass-white clover urine; S_{pl}= plantain sward; U_{pl} = plantain urine.

4.4.6 Nitrogen uptake and dry matter yield by the swards

In spring 2017, urine from cows fed ryegrass-white clover increased (P = 0.04) the dry matter (DM) yield compared to plantain urine. However, there were no difference (P = 0.09) in the DM yield between sward treatments (Table 4.5). In the autumn/winter 2018 experiment, the DM yield was similar (*P*= 0.60) between both urine treatments and both swards (*P*= 0.09).

Table 4.5. Cumulative pasture DM (kg ha^{-1}), N uptake (kg N ha^{-1}) by pastures and N concentration (%) in the swards, in spring 2017 (40 days) and autumn/winter 2018 (83 days) (n=5). (Values are mean ± SEM).

	DM (kg ha^{-1})		**N uptake (kg ha^{-1})**	
Treatments	Spring 2017	Autumn/ Winter 2018	Spring 2017	Autumn/ winter 2018
$S_{r/w}$_$U_{r/w}$	2571 ± 260	1155 ± 57	75 ± 10	37 ± 4
$S_{r/w}$_U_{pl}	2068 ± 168	1275 ± 83	55 ± 5	37 ± 4
$S_{r/w}$_Water	1868 ± 19	1045 ± 124	41 ± 2	16 ± 2
S_{pl}_$U_{r/w}$	2166 ± 306	1385 ± 87	61 ± 9	41 ± 3
S_{pl}_U_{pl}	1664 ± 121	1346 ± 121	42 ± 4	33 ± 5
S_{pl}_Water	2230 ± 173	1104 ± 188	44 ± 4	23 ± 5
Significance				
Sward type	0.09	0.09	0.09	0.73
Urine type	0.04	0.60	0.02	0.24
Interaction	0.99	0.31	0.94	0.51

Nitrogen uptake by the plantain and ryegrass-white clover swards increased with urine application (Table 4.5). In spring 2017, N uptake by the swards was not different (*P*= 0.09), however, when ryegrass-white clover urine was applied the N uptake was greater (*P* = 0.02) than for plantain urine. In the autumn/winter 2018 experiment, N uptake was not different regardless of the sward (*P*= 0.73) or urine type (*P*=0.24).

4.5 DISCUSSION

In the current experiment, plantain reduced N losses from the system through both a urine and a sward effect. A reduction in the urea-N concentration in urine of cows fed plantain swards was observed in spring 2017 and autumn/winter 2018 compared to urine from cows fed ryegrass-white clover swards. Decreasing total N and/or urea-N concentrations in urine of cows is a proposed strategy to reduce the N_2O emissions from urine patches in grazed pastoral system (Dijkstra *et al*., 2013). However, the effects reported of urine N loading rate on N_2O emissions factors are not consistent (de Klein *et al*. 2014). Di *et al*. (2016) showed that the N_2O emission factors increased from 0.7% to 1.1% when the N load in cow urine patches rose 29%, from 500 to 700 kg N ha^{-1}. de Klein *et al*. (2014) observed that on soils with poor drainage and high N_2O emissions, urine N concentrations and associated N loads did not affect the N_2O emission factors.

The reduction in the urea-N concentration in the urine of cows fed plantain swards is associated with the effect of plantain in the animal. Box *et al*. (2017) observed reductions in urea-N and total N concentrations in cows' urine, equal to 57 and 56%, and 61 and 53% in autumn and spring, respectively, when cows were fed plantain compared to ryegrass-white clover swards. The reductions in urea-N concentration in the urine of cows grazing plantain could be associated with a dilution effect and a reduction in the proportion of the N ingested in plantain that is partitioned to urine (Minnée *et al*., 2020; Navarrete *et al*., 2019; Totty *et al*., 2013). Studies where plantain was incorporated into the animal's diet suggest that more N could be partitioned to dung, reducing urinary N concentration which could reduce the cumulative N_2O emissions from urine patches.

A dilution effect when plantain was incorporated into the cows' diet has been observed, reducing the urine N and urea-N concentrations (Al-Marashdeh *et al*., 2019; Box *et al*., 2017; Mangwe *et al*., 2019; Navarrete *et al*., 2019). According to Mangwe *et al*. (2019), an increase in the total daily urine volume was more related to urination frequency than urine volume per event. An increase in urination frequency by cows grazing plantain would increase the area covered by urine patches and potentially increase the cumulative N_2O emissions due to an increase in urine patches at the paddock scale. However, if the total amount of urine N excreted by cows grazing plantain did not increase, the total N_2O emitted by urine of cows grazing plantain will not increase (de Klein *et al*., 2019). This mechanism is still unclear and further research is required to understand how an increase in the urination frequency might affect N_2O emissions from urine patches.

The secondary metabolites produced by plantain could also affect the urea-N concentration in urine of cows grazing plantain. Navarrete *et al.* (2016) reported that aucubin, a secondary metabolite, had an inhibitor effect on rumen fermentation, reducing the rumen ammonia production which could potentially reduce the urea-N concentration in the urine of cows fed plantain pastures. In the current study, aucubin concentrations in plantain leaves were higher in spring 2017 than autumn 2018 (results from Chapter 3); and the urine effect on N_2O emissions was observed in the autumn/winter 2018 experiment.

Despite that urea-N concentration in the urine of cows fed plantain was 45% lower (629 mg urea-N L^{-1}) than in the urine of cows grazing ryegrass-white clover in spring 2017, no effect of the urine of cows grazing plantain on the cumulative N_2O emissions and emission factors was observed. In contrast to these results, in autumn/winter 2018, both the cumulative N_2O emissions and emission factors were lower in the urine of cows fed plantain compared to the urine of cows fed ryegrass-white clover. In autumn/winter 2018, the urea-N in the urine of cows grazing plantain was reduced by 40% (1467 urea-N mg L^{-1}) compared to the urine of the ryegrass-white clover treatment. Simon *et al.* (2019) also observed an urine effect on N_2O emissions when the urine of cows grazing plantain was applied to ryegrass-white clover pastures in autumn.

A lower urea-N concentration in urine can also be associated with lower NH_3 volatilisation from urine patches (Zaman & Blennerhassett, 2010). In both spring and autumn/winter of this study, the NH_3 volatilisation from the urine of cows fed plantain was 80-90% lower than from the urine of cows fed ryegrass-white clover, respectively, in both swards. This urine effect on NH_3 losses could be associated with the reduced urea-N concentration in the urine of cows grazing plantain compared to urine of cows fed ryegrass-white clover. Recently, Hedges *et al.* (2020) reported an increase in total NH_3 emissions when the spread area of a cow urine patch increased from 0.25 to 1 m^2. However, an increase in NH_3 losses could have the potential of reducing soil NO_3^- concentrations, decreasing NO_3^- leaching and N_2O emissions. The effect of increasing the area covered by the urine patch on N_2O losses is still unclear and further work is needed to understand this process.

In spring 2017, the lack of difference in the soil NH_4^+ concentration under the plantain and ryegrass-white clover derived urine could suggest more urea in plantain urine was hydrolysed by urease into NH_4^+ in the soil (Zaman *et al*., 2009). However, in autumn/winter 2018, greater concentrations of soil NH_4^+ were observed when the urine from cows fed ryegrass-white clover swards was applied which was attributed to the higher urea-N concentration in ryegrass-white clover urine than plantain urine.

In spring 2017, the plantain sward reduced the cumulative N_2O emissions by 31% compared to ryegrass-white clover sward. A reason explaining the lower N_2O fluxes from the plantain sward in spring was probably a reduction in the nitrification process in the soil under the plantain sward. Studies have suggested that plantain can produce secondary metabolites; acteoside, aucubin and catalpol (Stewart, 1996; Tamura & Nishibe, 2002). Aucubin could potentially reduce the nitrification process (Dietz *et al*., 2013) by a mechanism described as biological nitrification inhibition (BNI) (Subbarao *et al*., 2007, 2013). In this mechanism, aucubin can inactivate the ammonia monooxygenase (AMO) enzyme which is responsible for the conversion of NH_4^+ into NO_2^-, therefore, decreasing the NO_3^- concentration in the soil. Luo *et al*. (2018) and Simon *et al*. (2019) found that when urine from cows fed ryegrass-white clover was applied to a plantain sward, cumulative N_2O fluxes were reduced by 74% and 44%, respectively. However, how aucubin affects N_2O emissions is still not clear and there is no published study that has reported the presence of aucubin in root exudates from plantain and in the urine of cows grazing plantain.

No effect of the plantain sward on soil NH_4^+ and NO_3^- concentrations was found in spring 2017, which does not support the hypothesis that plantain excretes BNI compounds in the root system. Simon *et al*. (2019) did not observe any differences in soil mineral N when ryegrass-white clover urine was applied onto swards containing different proportion of plantain (0, 30, 60 and 100%). However, in a laboratory experiment, Dietz *et al.* (2013) observed that applying a plantain leaf extract, and aucubin and catalpol at a rate of 0.6 mg g^{-1} soil, decreased the soil NO_3^- concentration and increased the soil NH_4^+ concentration. In the current experiment, there was not a sward effect on NH_3

volatilisation. In previous studies, when the nitrification inhibitor DCD was applied to the soil there was an increase in NH_3 losses and a reduction in N_2O emissions, due to an accumulation of NH_4^+ and an increase in soil pH (Zaman *et al*., 2009, 2013). In the current experiment, the ability of the plantain sward to produce biological nitrification inhibitors was not clear in the soil mineral N concentration.

In the autumn/winter experiment 2018, the plantain sward increased N_2O emitted from both urine treatments relative to the ryegrass-white clover sward. During this season, N_2O emission factors were 3.7 times higher in the plantain sward than in the ryegrass-white clover sward. The three reasons that may explain the increase in N_2O emissions by the plantain sward are: a) the effect of plantain sward on WFPS; b) the effect of bare soil on N_2O emissions (Bowatte *et al*., 2018); and c) an increase in soil NO_3^- concentrations.

An increase in WFPS can lead to an increase in anaerobic sites which would result in high denitrification rates increasing N_2O emissions (Saggar *et al*., 2013). In a short experiment carried out in late autumn/winter 2019, it was shown that the plantain sward treatment had greater WFPS than the ryegrass-white clover sward on two sampling days. This contrasted with the result reported by Luo *et al*. (2018) who found that soil WFPS values were about 6% lower in the plantain sward than the ryegrass-white clover during winter/spring 2015. However, that experiment was carried out on a well-drained Horotiu silt loam, while the soil in the current study, the Tokomaru silt loam, is fine textured, prone to surface soil compaction and is frequently wet at the surface even when mole and pipe drainage system have been installed. Lower plant density in the plantain sward could result in an increase in N_2O emissions from the plantain sward. Bowatte *et al*. (2018) found that the presence of plants reduced N_2O emissions compared to bare ground. In the autumn/winter experiment, there was more uncovered soil surface in the plantain swards due to the growth pattern of plantain, therefore, there was less potential to N uptake by pasture and, greater potential for N_2O emissions.

Soil NO_3^- concentrations under the plantain sward were greater than under ryegrass-white clover in three sampling days in autumn/winter. This difference could be attributed to the low plant density of plantain in the soil (Bowatte *et al*. 2018), decreasing the NO_3^- uptake by the pasture. Another explanation could be that during autumn/winter plantain is not actively growing (Stewart, 1996), and less NO_3^- is taken up from soils. This could explain the higher N_2O emissions from plantain sward than ryegrass-white clover. High soil NO_3^- concentrations could result in more N available for denitrification increasing N_2O emissions under anaerobic conditions.

In these experiments, plantain reduced N_2O emissions through a reduction in the urea-N concentration in the urine of cows fed plantain sward (autumn/winter 2018) and a sward effect, that could reduce the nitrification process in soils (spring 2017). Further work is required to understand the effect of aucubin in the nitrification process and determine if this metabolite is released by the root system of plantain.

4.6 CONCLUSIONS

Plantain decreased N_2O emission, relative to perennial ryegrass-white clover, by two mechanisms. In spring the plantain sward decreased N_2O emissions regardless of urine source, probably due to the bioactivity of aucubin. In autumn/winter cows grazing plantain produced urine with a lower N concentration, which resulted in decreased N_2O emissions from urine patches. However, N_2O emissions were greater from plantain swards, irrespective of source of urine applied, in autumn/winter due to greater water filled pore space. Ammonia volatilisation was always lower from plantain urine than ryegrass-white clover urine.

4.7 REFERENCES

Al-Marashdeh, O., Judson, H., Schetelat, S., Pawsey, A. & Edwards, G. (2019). Urinary nitrogen excretion and water balance of dairy heifers fed cut plantain or perennial-ryegrass pastures. *New Zealand Journal of Animal Science and Production*, *79,* 171-173.

Baggs, E. M. & Philippot, L. (2010). Microbial terrestrial pathways to nitrous oxide. In K. A. Smith (Ed.), *Nitrous oxide and climate change* (pp. 4-35). London: Earthscan.

Blakemore L. C. (1987). Methods for chemical analysis of soils. *NZ Soil Bur Sci Rep*., *80*, 71–76.

Bolan, N. S., Saggar, S., Luo, J., Bhandral, R. & Singh, J. (2004). Gaseous emissions of nitrogen from grazed pastures: processes, measurements and modelling, environmental implications, and mitigation. *Advances in agronomy, 84*(37), 120.

Bowatte, S., Hoogendoorn, C. J., Newton, P. C. D., Liu, Y., Brock, S. C. & Theobald, P. W. (2018). Grassland plant species and cultivar effects on nitrous oxide emissions after urine application. *Geoderma*, *323*, 74-82.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2017). Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research, 60*(4), 470-482.

Bremner, J. M. (1997). Sources of nitrous oxide in soils. *Nutrient Cycling in Agroecosystems, 49*(1), 7-16.

Castillo, A., Kebreab, E., Beever, D., & Ja, F. (2000). A review of efficiency of nitrogen utilisation in lactating dairy cows and its relationship with environmental pollution. *Journal of Animal and Feed Sciences, 9*, 1-32.

Christensen, C., Hedley, M. J., Hanly, J. A. & Horne, D. J. (2018). Duration-controlled grazing of dairy cows. 2: nitrogen losses in sub-surface drainage water and surface runoff. *New Zealand Journal of Agricultural Research*, *62,* 48-68.

de Klein, C. A. M., Barton, L., Sherlock, R. R., Li, Z. & Littlejohn, R.P. (2003). Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. *Soil Research*, *41*(3), 381-399.

de Klein, C. A. M., Luo, J., Woodward, K. B., Styles, T., Wise, B., Lindsey, S. & Cox, N. (2014). The effect of nitrogen concentration in synthetic cattle urine on nitrous oxide emissions. *Agriculture, Ecosystems & Environment, 188,* 85-92.

de Klein, C. A. M., van der Weerden, T. J., Luo, J., Cameron, K. C. & Di, H. J. (2019). A review of plant options for mitigating nitrous oxide emissions from pasture-based systems. *New Zealand Journal of Agricultural Research, 63*(1), 29-43.

Di, H. J. & Cameron, K. C. (2002). The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland. *Soil Use and Management, 18*(4), 395-403.

Di, H.J. & Cameron, K. C. (2016). Inhibition of nitrification to mitigate nitrate leaching and nitrous oxide emissions in grazed grassland: a review. *J. Soils Sediments 16,* 1401–1420.

Di, H. J., Cameron, K. C., Podolyan, A., Edwards, G. R., de Klein, C. A. M., Dynes, R. & Woods, R. (2016). The potential of using alternative pastures, forage crops and gibberellic acid to mitigate nitrous oxide emissions. *Journal of Soils and Sediments*, *16*(9), 2252-2262.

Dietz, M., Machill, S., Hoffmann, H. C. & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil, 368*(1-2), 445-458.

Dijkstra, J., Oenema, O., Van Groenigen, J. W., Spek, J. W., Van Vuuren, A. M. & Bannink, A. (2013). Diet effects on urine composition of cattle and N_2O emissions. *Animal*, *7*, 292-302.

Douglas, L. A. & Bremner, J. M. (1970). Extraction and Colorimetric Determination of Urea in Soils *Soil Science Society of America Journal, 34*(6), 859-862.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019). Efficacy of aucubin as a nitrification inhibitor assessed in two Canterbury field trials. *New Zealand Journal of Agricultural Research*, *63*(1),73-86.

Haynes, R. J., & Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in agronomy, 49*, 119-99.

Hedges M. T., Hanly, J. A., Horne, D. J. & Saggar, S. (2020). Effect of increasing cow urine patch area on ammonia emissions in a pasture soil. In: Nutrient management in Farmed Landscapes. (Eds C. L. Christensen, D. J. Horne and R. Singh). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 33. Farmed

Landscapes Research Centre, Massey University, Palmerston North, New Zealand. 5 pages.

Hewitt, A. E. (2010). *New Zealand soil classification 3rd edition - Lincoln, NZ*: Manaaki Whenua press, 2010.

IPCC. (2014). Climate change 2014: synthesis report. Core Writing Team. In: Pachauri, R.K., Meyer, L. (Eds.), Fifth Assesment Report. 1–151 (Geneva, Switzerland).

Kissel, D. E., Brewer, H. L. & Arkin, G. F. (1977). Design and test of a field sampler for ammonia volatilization. *Soil Science Society of America Journal, 41*(6), 1133-1138.

Luo, J., Balvert, S. F., Wise, B., Welten, B., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. & Judge, A. (2018). Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. *Science of The Total Environment, 610–611*, 1271-1280.

Luo, J., de Klein, C. A. M., Ledgard, S. F. & Saggar, S. (2010). Management options to reduce nitrous oxide emissions from intensively grazed pastures: A review. *Agriculture, Ecosystems & Environment*, *136*(3–4), 282-291.

Luo, J., Hoogendoorn, C., van der Weerden, T., Saggar, S., de Klein, C. A.M., Giltrap, D., Rollo, M. & Rys, G. (2013). Nitrous oxide emissions from grazed hill land in New Zealand. *Agriculture, Ecosystems & Environment*, *181*, 58-68.

Mangwe, M. C., Bryant, R. H., Beck, M. R., Beale, N., Bunt, C. & Gregorini, P. (2019). Forage herbs as an alternative to ryegrass-white clover to alter urination patterns in grazing dairy systems. *Animal Feed Science and Technology, 252*, 11-22.

McKenzie, H. & Wallace, H. S. (1954). The Kjeldahl determination of nitrogen: a critical study of digestion conditions-temperature, catalyst, and oxidizing agent. *Australian Journal of Chemistry*, *7*(1), 55-70.

Minnée, E. M. K., Leach, C. M. T. & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science*, *239*, 104093.

Ministry for the Environment & Stats NZ. (2019a). New Zealand’s Environmental Reporting Series: Environment Aotearoa 2019. Available from www.mfe.govt.nz and www.stats.govt.nz.

Ministry for the Environment. (2019b). Available from https://www.mfe.govt.nz/climate-change/why-climate-change-matters/global-response/paris-agreement

Ministry for the Environment. (2019c). Climate Change Response (Zero Carbon) Amendment Bill: Summary. Wellington: Ministry for the Environment. ISBN: 978-1-98-857926-9. Publication number: ME 1410.

Misselbrook, T. H., Del Prado, A. & Chadwick, D. (2013). Opportunities for reducing environmental emissions from forage-based dairy farms. *Agricultural and Food Science*, 22(1), 93-107.

Misselbrook, T. H., Powell, J. M., Broderick, G. A. & Grabber, J. H. (2005). Dietary Manipulation in Dairy Cattle: Laboratory Experiments to Assess the Influence on Ammonia Emissions. *Journal of Dairy Science, 88*(5), 1765-1777.

Mulvaney, R. L. & Bremner, J. M. (1979). A modified diacetyl monoxime method for colorimetric determination of urea in soil extracts. *Communications in Soil Science and Plant Analysis, 10*(8), 1163-1170.

Navarrete, S., Kemp, P. D., Pain, S. J. & Back, P. J. (2016). Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata* L.) and their effect on *in vitro* rumen fermentation. *Animal Feed Science and Technology*, *222*, 158-67.

Navarrete, S., Kemp, P. D., Rodriguez, M. J., Hedley, M. J., Horne, D. J. & Hanly, J. A. (2019). Decreased nitrate leaching when lactating cows graze plantain (*Plantago lanceolata*) pastures. Paper presented at the Land Use and Water Quality: Agriculture and the Environment, Aarhus, Denmark.

Oenema, O., Velthof, G. L., Yamulki, S. & Jarvis, S. C. (1997). Nitrous oxide emissions from grazed grassland. *Soil Use and Management, 13*(s4), 288-295.

Petersen, S. O., Sommer, S. G., Aaes, O. & Søegaard, K. (1998). Ammonia losses from urine and dung of grazing cattle: effect of N intake. *Atmospheric Environment, 32*(3), 295-300.

Pijlman, J., Berger, S. J., Lexmond, F., Bloem, J., van Groenigen, J. W., Visser, E. J. W., Erisman, J. W. & van Eekeren, N. (2019). Can the presence of plantain (*Plantago lanceolata* L.) improve nitrogen cycling of dairy grassland systems on peat soils? *New Zealand Journal of Agricultural Research, 63*(1), 106-122.

Ravishankara, A. R., Daniel, J. S. & Portmann, R. W. (2009). Nitrous Oxide (N_2O): The Dominant Ozone-Depleting Substance Emitted in the 21st Century. *Science, 326*(5949), 123-125.

Rodriguez, M. J., Saggar, S., Berben, P., Palmada, T., Lopez-Villalobos, N. & Pal, P. (2019). Use of a urease inhibitor to mitigate ammonia emissions from urine patches. *Environmental Technology*.

Ryden, J. C., Whitehead, D. C., Lockyer, D. R., Thompson, R. B., Skinner, J. H. & Garwood, E. A. (1987). Ammonia emission from grassland and livestock production systems in the UK. *Environmental pollution, 48*(3), 173-184.

Saggar, S., Bolan, N. S. & Singh, J. (2005). Economic and environmental impacts of increased nitrogen use in grazed pastures and role of inhibitors in mitigating nitrogen losses. *New Zealand Science Review, 62*(3), 62-67.

Saggar, S., Jha, N., Deslippe, J., Bolan, N. S., Luo, J., Giltrap, D., Kim, D-G., Zaman M. & Tillman, R. W. (2013). Denitrification and N_2O:N_2 production in temperate grasslands: Processes, measurements, modelling and mitigating negative impacts. *Science of The Total Environment, 465,* 173-195.

Scotter, D. R., Cothier, B. E. & Turner, M. A. (1979). The soil water balance in a gragiaqualf and its effect on pasture growth in central New Zealand. *Soil Research, 17*(3), 455-465.

Selbie, D. R., Buckthought, L. E. & Shepherd, M. A. (2015). The Challenge of the Urine Patch for Managing Nitrogen in Grazed Pasture Systems. *Advances in agronomy, 129,* 229-292.

Simon, P. L., de Klein, C. A. M., Worth, W., Rutherford, A. J. & Dieckow, J. (2019). The efficacy of Plantago lanceolata for mitigating nitrous oxide emissions from cattle urine patches. *Science of The Total Environment*, *691*, 430-441.

Stewart, A. V. (1996). Plantain (*Plantago lanceolata* L.): A potential pasture species. *Proceedings of the New Zealand Grassland Association 58,* 77-86.

Subbarao, G. V., Rao, I. M., Nakahara, K., Sahrawat, K. L., Ando, Y. & Kawashima, T. (2013). Potential for biological nitrification inhibition to reduce nitrification and N_2O emissions in pasture crop–livestock systems. *Animal, 7*(s2), 322-332.

Subbarao, G. V., Rondon, M., Ito, O., Ishikawa, T., Rao, I. M., Nakahara, K., Lascano, C. & Berry, W. L. (2007). Biological nitrification inhibition (BNI)—is it a widespread phenomenon? *Plant and Soil, 294*(1-2), 5-18.

Tamura, Y. & Nishibe, S. (2002). Changes in the Concentrations of Bioactive Compounds in Plantain Leaves. *Journal of Agricultural and Food Chemistry, 50*(9), 2514-2518.

Totty, V. K., Greenwood, S. L., Bryant, R. H. & Edwards, G. R. (2013). Nitrogen partitioning and milk production of dairy cows grazing simple and diverse pastures. *Journal of Dairy Science, 96*(1), 141-149.

van der Weerden, T. J., Clough, T. J. & Styles, T. M. (2013). Using near-continuous measurements of N2O emission from urine-affected soil to guide manual gas sampling regimes. *New Zealand Journal of Agricultural Research, 56*(1), 60-76.

Zaman, M. & Blennerhassett, J. D. (2010). Effects of the different rates of urease and nitrification inhibitors on gaseous emissions of ammonia and nitrous oxide, nitrate leaching and pasture production from urine patches in an intensive grazed pasture system. *Agriculture, Ecosystems & Environment, 136*(3–4), 236-246.

Zaman, M., Saggar, S., Blennerhassett, J. D. & Singh, J. (2009). Effect of urease and nitrification inhibitors on N transformation, gaseous emissions of ammonia and nitrous oxide, pasture yield and N uptake in grazed pasture system. *Soil Biology and Biochemistry, 41*(6), 1270-1280.

Zaman, M., Zaman, S., Nguyen, M. L., Smith, T. J. & Nawaz, S. (2013). The effect of urease and nitrification inhibitors on ammonia and nitrous oxide emissions from simulated urine patches in pastoral system: A two-year study. *Science of The Total Environment, 465*(Supplement C), 97-106.

5 AUCUBIN: A BIOLOGICAL NITRIFICATION INHIBITOR FOR REDUCING NITROGEN LOSSES FROM URINE PATCHES

5.1 ABSTRACT

Plantain (*Plantago lanceolata* L.) is a forage that produces secondary metabolites with the potential to inhibit the soil nitrification process. This study aimed to i) quantify the release of aucubin and catalpol by plantain root systems; ii) evaluate the effect of aucubin on nitrogen (N) leaching and nitrous oxide (N_2O) emissions from urine of cows fed ryegrass-white clover applied to plantain and ryegrass-white clover swards; and, iii) compare N losses from urine of cows fed plantain compared to urine from cows fed ryegrass-white clover when applied to a ryegrass-white clover sward. A hydroponic experiment was conducted in a glasshouse and the root exudates of plantain and ryegrass were periodically analysed. Lysimeter studies were used to measure N leaching and N_2O emissions from a plantain sward compared to a ryegrass-white clover sward after aucubin and urine were applied. Nitrogen losses were also determined from the two urine treatments adjusted to the same N load, and then applied to lysimeters with ryegrass-white clover swards. The hydroponic experiment suggests that aucubin was not released by the roots of plantain. Nitrous oxide emissions were reduced by 34% when aucubin was applied to the ryegrass-white clover sward. Aucubin had no effect on NO_3^- leaching. Urine from cows grazing the plantain sward had no effect on N_2O emissions, and N leaching when compared to the ryegrass-white clover urine with the same N loading rate. Nitrous oxide emissions from urine patches are decreased by aucubin released into the soil from plantain plants, probably via leaf litter.

Keywords: plantain; secondary metabolites; nitrous oxide; nitrate leaching; dairy systems

5.2 INTRODUCTION

Urine patches are the largest source of nitrogen (N) losses from grazed dairy pastures to the wider environment and can cover between 4 to 29% of the grazed pasture area per year (Haynes & Williams, 1993; Moir *et al.*, 2011). The N concentration in a cow urine patch can be up to 1000 kg N ha^{-1} (Di & Cameron, 2002a; Haynes & Williams, 1993) in a small area, which exceeds immediate plant requirements (Ledgard *et al.*, 2009). Nitrogen not taken up by plants is susceptible to loss to the environment as nitrous oxide (N_2O) and nitrate (NO_3^-) leaching (Cameron *et al.*, 2013; Stark & Richards, 2008).

A novel strategy to reduce N_2O emissions and NO_3^- leaching from urine patches is the use of alternative forage species to decrease N concentrations in cow urine. Plantain (*Plantago lanceolata* L.) has been identified as a forage species that can reduce N concentration in cows' urine (Box *et al.*, 2017; Minnée *et al.*, 2020) , and also potentially release secondary metabolite from root exudates that could reduce nitrification rates in the soil (de Klein *et al.*, 2019; Dietz *et al.*, 2013), and consequently N losses to the environment.

Aucubin, a well-known secondary metabolite produced by plantain, has been identified as a biological nitrification inhibitor (Bartholomaeus & Ahokas, 1995; Davini *et al.*, 1986; Gardiner *et al.*, 2016). Aucubigenin, the aglycone of aucubin, which can inhibit cytochrome P-450, and aucubigenin's structure indicates that it can inhibit the enzyme ammonia monooxygenase (AMO) in soil, thereby inhibiting the first step of the nitrification pathway (Bartholomaeus & Ahokas, 1995; Davini *et al.*, 1986). Soil incubation experiments and field experiments adding plantain leaf extract or commercial aucubin found an inhibition of nitrification (Dietz *et al.*, 2013; Gardiner *et al.*, 2017).

Lysimeters and field studies have shown that swards that include plantain decreased NO_3^- leaching and N_2O emissions from urine patches compared to ryegrass-white clover swards, confirming that there is a sward effect (Carlton *et al.*, 2018; Luo *et al.*, 2018; Simon *et al.*, 2019; Welten *et al.*, 2019; Woods *et al.*, 2018). Judson *et al.* (2019) showed that urine from cows fed plantain sward also inhibits the soil nitrification process, and they suggested that the BNI compounds could be present in the cows' urine. A soil incubation experiment, comparing the effect of cows' urine from cows that were grazing either plantain or ryegrass-white clover pastures applied to a Italian ryegrass swards, showed that the plantain urine reduced soil nitrification over a 30 day period, compared to ryegrass-white clover urine, even though both types of urine had the same total N concentration (Judson *et al*., 2019). This result suggests that another factor, other than urine N concentration, could explain the decrease in soil nitrification. The effect of secondary metabolites, present in the plantain urine, would have potential to explain the observed reduction in nitrification (Judson *et al.*, 2019), but, to our knowledge the presence of the secondary metabolites in urine has not been quantified. Gardiner *et al.* (2019b) evaluated the effectiveness of aucubin at reducing N_2O emissions, reporting that aucubin inhibited nitrification for a short period of time, from day 5 to 17 after urine application to soil. There is limited information on the application of aucubin to soil, immediately before urine deposition, on both N_2O emissions and NO_3^- leaching at the same time. The current experiment will evaluate the effect of aucubin applied to two swards, plantain and ryegrass-white clover.

This study was divided into two experiments. In the first experiment, the production of secondary metabolites by plantain roots was assessed. The second experiment determined the effect of the application of pure aucubin on N_2O emissions and NO_3^- leaching losses, following urine deposition onto the soil. The following effects were evaluated: a) the effect of aucubin on N_2O and NO_3^- losses from ryegrass-white clover and plantain swards when aucubin was applied before urine; and b) the effect of urine from cows grazing plantain on N_2O and NO_3^- losses from a ryegrass- white clover sward. It was hypothesised that: i) plantain may release key secondary metabolites (i.e. aucubin and catalpol) from its root system; ii) aucubin application will reduce N_2O emissions and NO_3^- leaching from both ryegrass-white clover and plantain swards; and iii) urine from cows grazing plantain will reduce N_2O emissions and NO_3^- leaching, compared with urine from cows grazing ryegrass-white clover swards, even when both types of urine have the same N concentration. The first hypothesis was tested in a hydroponic experiment and the last two hypotheses were tested in a lysimeter experiment.

5.3 MATERIALS AND METHODS

5.3.1 Hydroponic experiment

A hydroponic experiment was conducted in a glasshouse at Massey University from 14th January to 29th of April 2019. Two cultivars of plantain (cv "Ceres Tonic" and "Agritonic") and one of perennial ryegrass (cv "Trojan") as a control, were grown in a glasshouse to quantify the production of key plantain secondary metabolites (catalpol and aucubin).

The experiment consisted of nine tanks (3 replicate tanks for each plant treatment) with 15 plants of the same plant type in each tank. During the sampling days, five plants were removed per tank and the root exudates were collected.

Seeds of plantain and ryegrass were sown in a sand vermiculite mixture (3:1) and grown for 10 days (Figure 5.1a). After ten days, the seedlings were transferred to the hydroponic system (28th of January) and grown in 40 L tanks (370 x 550 x 200 mm) on floating styrofoam blocks (Figure 5.1b). The nutrient solution consisted of nutrient concentrations that are described in Subbarao *et al*. (2007). The solution was replaced every week and the pH of the solution was adjusted to 5.8 – 6.0 weekly. The N source in the solution was 1mM N as $(NH_4)_2SO_4$.

Figure 5.1. Hydroponic experiment, a) plantain and ryegrass seedlings growing in a sand:vermiculite mixture (3:1) for 10 days; b) seedlings were transferred to the hydroponic system; c) plants 40 days after being transferred to the hydroponic system, d) plantain and e) ryegrass plants sampled for root exudates after 40 days.

After plants had spent 40, 50 and 60 days in the hydroponic system (Subbarao *et al*., 2007) (Figure 5.1c), the root exudates were collected from intact plants (Figure 5.1 d and e). Root exudate from plants was collected destructively, the plants removed from the nutrient solutions were not returned to the solutions. At each sampling time, each plant's roots were briefly washed in tap water and then immersed for 1 hour in 1 L of 0.5 mM $(NH_4)_2SO_4$, as a pre-treatment before the collection. Roots were then washed with de-ionized water, then nano-water and finally, immersed in 1 L of aerated nano-water, as the medium to collect the exudates for 24 hours (Subbarao *et al*., 2007). The root exudates from each plant were frozen until the extraction. The dry weight of roots and leaves was measured on each sampling day. Those samples were oven dried at 70 ^{o}C for 48 h.

To extract the bioactive compounds, the exudate was evaporated to dryness using a rotary evaporator under vacuum at 40 °C, and then extracted with 30 ml of methanol. Then, the methanol extract was evaporated to dryness using a vacuum concentrator at 35 °C. Catalpol and aucubin in the root exudates and leaves from plantain and ryegrass were determined by high-performance liquid chromatography (HPLC). Commercially available catalpol (99% pure; Extrasynthese S.A, France) and aucubin (99% pure; Xian Plant Bio-Engineering Co., Ltd, China) were used as standards. High-performance liquid chromatography was performed at 40 °C using a 100 mm × 6.0 YMC pack ODS-A column protected by a YMC guard pack (YMC America, Inc). The mobile phase was 1% acetronitrile in water for catalpol and aucubin. The flow rate was 1 mL/min. Wavelength detection was performed at 240 nm for catalpol and aucubin. The HPLC system consisted of a Dionex UltiMate 3000 HPLC system equipped with an UltiMate 3000 Pump, an UltiMate 3000 Autosampler Column Compartment, an UltiMate 3000 variable wavelength detector and Chromeleon software (version 6.8) for data processing.

5.3.2 Lysimeter experiment

5.3.2.1 Experimental design

A lysimeter experiment was conducted in late autumn/winter (29th March 2019 to 5th July 2019) in order to investigate the effects of aucubin and pasture species on N_2O emissions and NO_3^- leaching. The experiment evaluated two sward treatments (ryegrass-white clover and pure plantain) without and with aucubin (concentration equivalent to 10 mg g^{-1} DM plantain leaves which equated to 11.4 kg ha^{-1}). All swards received urine from cows grazing ryegrass-white clover pasture. The N in the urine was applied at a rate of 583 kg N ha^{-1}. A water control with and without aucubin applied was also included. The treatments used are detailed in Table 5.1.

To determine the effect of urine composition on N_2O emissions and NO_3^- leaching, urine from cows grazing ryegrass-white clover and plantain swards were applied to ryegrass-white clover swards at equal N application rate of 449 kg N ha^{-1} (Table 5.1). The lysimeters containing plantain swards had at least two plants of plantain, however, the sward composition was not determined in this experiment. The treatments were replicated 5 times giving a total of 50 lysimeters.

5.3.2.2 Lysimeter collection

Lysimeters were collected from areas growing ryegrass-white clover and pure plantain swards established in December 2016 at Massey University's Dairy 4 Farm, Palmerston North (40° 39' S; 175 ° 61' E). Fifty intact soil lysimeters (200 mm diameter x 250 mm depth) were collected in March 2019 from the pre-established pure plantain and ryegrass-white clover swards using PVC tubes. Each PVC tube was gradually pushed down to 250 mm depth using a pneumatic hammer, and then dug out. The lysimeters were placed on tables that allowed for collection of drainage water. A plastic tube and a wick were connected to the bottom of each lysimeter and fed into a 2 L container for leachate collection.

Table 5.1. Treatments applied to the lysimeters. Abbreviations: wc – white clover, Auc - aucubin

Sward treatment	**Aucubin rate ($mg\ g^{-1}$ DM)**	**Urine Source**	**Treatment name**
Ryegrass-wc	0	Ryegrass-wc	$S_{r/w}$ _$U_{r/w}$
	10	Ryegrass-wc	$S_{r/w}$ _ $U_{r/w}$ + Auc
	0	Water	$S_{r/w}$_Water
	10	Water	$S_{r/w}$_Water+ Auc
Plantain	0	Ryegrass-wc	S_{pl} _$U_{r/w}$
	10	Ryegrass-wc	S_{pl} _ $U_{r/w}$ + Auc
	0	Water	S_{pl}_Water
	10	Water	S_{pl}_Water+ Auc
Ryegrass-wc	0	Plantain	$S_{r/w}$ _ U_{pl}
	0	Ryegrass-wc_diluted	$S_{r/w}$ _ $U_{r/wdil.}$

5.3.2.3 Treatments application

Aucubin (Xian Plant Bio-Engineering Co. Ltd, China) was applied at a rate of 10 mg g^{-1} DM of plantain leaves. The aucubin application rate was an average between the aucubin concentration in plantain leaves reported by Navarrete *et al.* (2016) and the rates found by Box & Judson (2018). The aucubin solution (19 g/L) application rate was 600 L ha^{-1}, which equated to 1.7 mL per lysimeter. Aucubin solution was sprayed onto the lysimeters before urine deposition.

Urine from cows grazing ryegrass-white clover and pure plantain swards was collected at Massey University Dairy 4 Farm, 24 hours before application, and kept at 4 ^{o}C to avoid urea hydrolysis. A sub-sample (100 mL) was analysed for total N and urea-N concentration. The urine of cows fed ryegrass-white clover was diluted with water to match the N concentration of the urine of cows fed pure plantain swards. Cow urine was applied at a rate of 10 L m^{-2} (Selbie *et al.*, 2015), and the control treatments received the same volume of water to ensure that the moisture inputs were consistent across all lysimeters.

5.3.2.4 N_2O measurements

Nitrous oxide emissions were monitored for 87 days following treatment application. Nitrous oxide samples were collected using the static chamber method. Each PVC chamber had an 0.20 m internal diameter and a height of 0.10 m, and a volume of 2.83 L. On each sampling day the chambers were placed in the top of the lysimeters and tight sealed. Then the chamber was covered by a wet towel to help to maintain a constant temperature inside the chambers. The PVC chambers used for gas sampling were sealed for 60 minutes and sampling occurred at 0, 30 and 60 minutes after the chamber was sealed. Gas samples (25 ml) were collected from a sampling port on the chamber, using a plastic syringe fitted with a three-way stopcock, and transferred to pre-evacuated 12 mL vials. Nitrous oxide sample collection was carried out between 11:00 am and 2:00 pm on each sampling day, which according to van der Weerden *et al.* (2013) allows extrapolation to a daily flux without bias. During the first month of the experiment, N_2O samples were taken twice a week and afterwards samples were taken weekly until the background level was reached. On each sampling day, three background air samples were also taken at each time interval.

Nitrous oxide concentrations were analysed via gas chromatography using a Shimadzu Nexis 2030 gas chromatograph. The emissions over the sampling time were integrated for each chamber to estimate the accumulated emissions during the measurement period (Luo *et al.*, 2013). The N_2O fluxes were calculated using the slope of the linear increase of N_2O concentration in the chamber over time (de Klein *et al.*, 2003), using the following equation:

$$N_2O\ \text{flux} = \frac{\delta N_2O}{\delta T} * \frac{M}{Vm} * \frac{V}{A} \tag{5.1}$$

where δ N_2O is the increase in N_2O in the headspace; δT is the enclosure period (hours); M is the molar weight of N in N_2O; Vm is the molar volume of the gas at the sampling temperature (L mol^{-1}); V is the headspace volume (m^3) and A is the area covered (m^2).

It was assumed that the ryegrass-white clover urine treatment without aucubin in ryegrass-white clover swards, would be the treatment that lost the most N-N_2O due to there not being any aucubin effect on N losses. Accordingly, the percentage reduction in total N as N_2O emitted for each treatment was calculated by comparing each treatment with the same treatment without aucubin. The total emissions over the experimental period were calculated by integrating the emissions per day over time. The N_2O emission factors (EF_3, N_2O-N emitted as % of N applied) were calculated using the following equation:

$$EF_3 = \frac{N_2O\text{ -N total (urine) -}N_2O\text{-N total (control)}}{\text{Urine N applied}} \tag{5.2}$$

where N_2O-N total (urine) and N_2O-N total (control) are the cumulative N_2O-N emissions for the measurement periods from the urine and control plots, respectively (kg N ha^{-1}), and Urine N applied is the rate of urine N applied (kg N ha^{-1}).

5.3.2.5 Nitrogen analyses in drainage and herbage

Over the experimental period leachate from each lysimeter was collected once per week, which involved measuring the volume of leachate and collecting a subsample for NO_3^-

and total N concentrations analyses. Filtered samples of leachate were analysed for NO_3^- using a Thermo Scientific Dionex Aquion Ion Chromatograph. Total N was determined on the unfiltered samples using the alkali persulphate digestion method of Hosomi and Sudo (1986). In this method, the different forms of N in the sample are converted to NO_3^- and analysed using the colorimetric methods on a Technicon Auto Analyser (Blakemore *et al.* 1987).

The herbage in each lysimeter was collected by cutting at a height of 50 mm above the soil surface once a month to mimic grazing and determine dry matter (DM) yield, N content and N uptake by pastures. The herbage samples were oven dried at 70 ^{o}C for 48 h to determine DM yield. The N content was determined by combustion using a Leco analyser (AOAC, 2000; method 968.06).

5.3.2.6 Rainfall and irrigation

The above ground temperature was monitored by temperature loggers (1 cm diameter) placed inside the lysimeters. The evapotranspiration (mm) was obtained from a meteorological station located close to the experimental site. Irrigation (mm) was applied during the first month of the experimental period using a manual sprinkler system. Every time irrigation was applied, the volume of water for each lysimeter was checked to ensure that the amount of water received was the same across all lysimeters. Containers of 50 ml were hooked to the lysimeter's wall to determine the amount of water received in each lysimeter. Rainfall (mm) was measured in situ using a rain gauge.

5.3.2.7 Statistical analysis

Data were analysed using the PROC MIXED procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC). Aucubin and catalpol in root exudates and leaves were analysed as a 2 x 2 factorial randomised design, with cultivars and days after transplantation as fixed effects and replicates as random effects. The EF_3 and drainage NO_3^- and total N leaching as well as N uptake and DM yield were analysed as a 2 x 2 factorial randomised design, with sward and aucubin rate as fixed effects and replicates as random effects. The effect of plantain sward on N_2O emissions was analysed with sward effect as fixed effect and replicates as random effect. The sward effect (ryegrass-white clover and plantain swards) was nested with the aucubin effect (0 and 10 mg g^{-1} aucubin). The normal distribution of the data were checked. Daily N_2O emissions data were log transformed to meet the assumptions of normality. For the comparison of cumulative N_2O, drainage NO_3^- and total N leaching from urine applications diluted to the same N concentration from cows fed plantain with cows fed ryegrass-white clover, , urine type was a fixed effect and replicates were random effects. Where significant effects existed, the LSD procedure at the 5% level was used to identify differences among means.

5.4 RESULTS

5.4.1 Hydroponic experiment

Aucubin was not detected in the root exudates during the experimental period (Table 5.2) but it was detected in the leaves of the two species and three cultivars, at each sampling day. Aucubin concentrations in plantain leaves were much greater than in ryegrass (Table 5.2). Moreover, aucubin concentrations in plantain leaves were similar between day 50 and 60, and these values were greater (*P*= 0.001) than in day 40 after transplantation (Table 5.2).

Catalpol was identified in the root exudates of each species 50 and 60 days after transplantation. There were no differences in catalpol concentrations between species, however, there was a tendency (*P*= 0.09) for greater catalpol concentration in the root exudates of plantain species. Catalpol was detected in the leaves of plantain 40 and 50 days after transplantation. After 60 days of transplantation catalpol was detected in the leaves of all the species, being greater (*P*= 0.03) in plantain leaves than leaves of ryegrass.

Table 5. 2. Catalpol and aucubin concentrations (ug L^{-1}) from root exudates and in the leaves (g kg^{-1} DM) in plantain (cv. Agritonic and Tonic) and ryegrass (cv. Trojan), 40, 50 and 60 days after transplantation (DAT) to the nutrient solution. (Values are mean ± SEM)

		Catalpol		**Aucubin**	
Species	**D.A.T**	**Root exudates (ug L^{-1})**	**Leaves (g kg^{-1} DM)**	**Root exudates (ug L^{-1})**	**Leaves (g kg^{-1} DM)**
Plantain cv. Agritonic	40	n.d.	0.2 ± 0.08	n.d.	4.0 ± 0.63
Plantain cv. Tonic	40	n.d.	0.1 ± 0.02	n.d.	3.9 ± 0.50
Ryegrass cv. Trojan	40	n.d.	-	n.d.	0.1 ± 0.02
Plantain cv. Agritonic	50	1.2 ± 0.15	0.5 ± 0.08	n.d.	13.1 ± 3.68
Plantain cv. Tonic	50	1.1 ± 1.01	0.2 ± 0.05	n.d.	11.0 ± 1.99
Ryegrass cv. Trojan	50	0.7 ± 0.43	-	n.d.	0.2 ± 0.02
Plantain cv. Agritonic	60	5.6 ± 3.17	0.4 ± 0.17	n.d.	10.4 ± 0.41
Plantain cv. Tonic	60	3.6 ± 1.22	0.3 ± 0.07	n.d.	10.8 ± 1.51
Ryegrass cv. Trojan	60	1.6 ± 0.61	0.04 ± 0.01	n.d.	0.1 ± 0.03
Significance					
Plant type		0.09	0.02		< 0.0001
DAT		0.45	0.42		0.001
Interaction		0.62	0.58		0.06

n.d. = not detected

5.4.2 Lysimeter experiment

5.4.2.1 Rainfall and irrigation

Daily rainfall (mm), irrigation (mm) and soil temperature (°C) during the experimental period (29th March 2019 to 5th July 2019) are shown on Figure 5.2. The daily mean air temperature ranged from -3 ºC to 27 ºC, and daily soil temperature (10 cm depth) ranged from 3 ºC to 19 ºC. Total water input for the experimental period was 492 mm, with 120 mm from irrigation and 372 mm from natural rainfall.

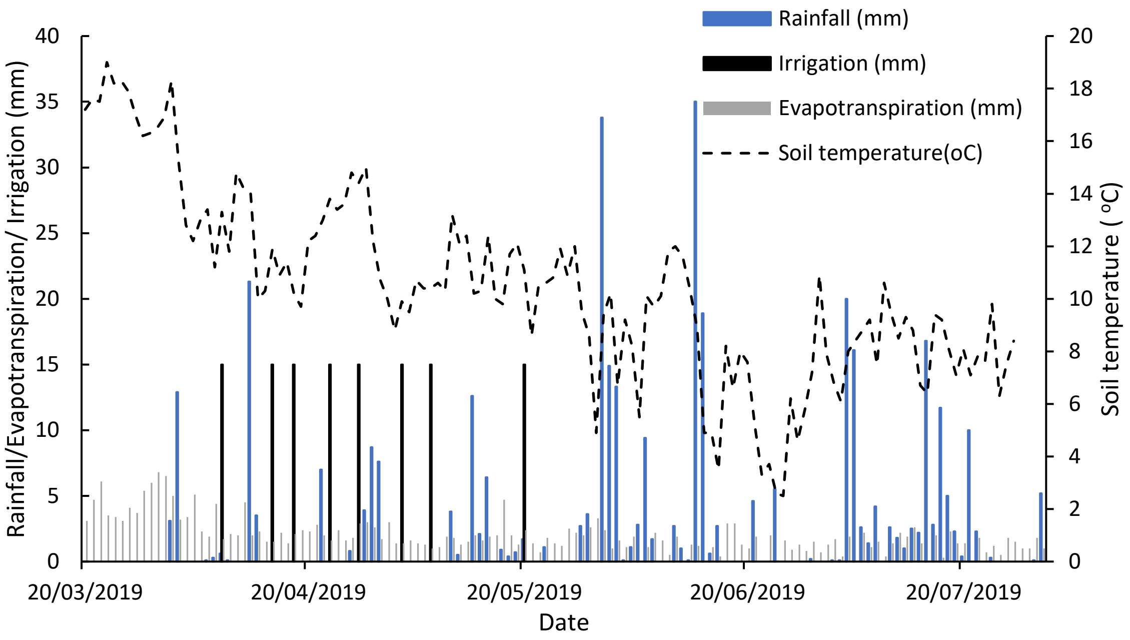


Figure 5.2. Daily precipitation (mm) (blue bars), irrigation (mm) (black bars), evapotranspiration (mm) (grey bars) and daily soil temperature (^{o}C) (dash line) during the experimental period.

5.4.2.2 Nitrous oxide emissions

Daily N_2O fluxes (kg N_2O-N ha^{-1} day^{-1}) from the lysimeters showed temporal variation, with emissions sharply increasing from all treatments after urine was applied (Figure 5.3). Nitrous oxide fluxes also varied with rainfall events. For the plantain (Figure 5.3a) and ryegrass-white clover swards (Figure 5.3b), urine application exhibited three N_2O peaks, with the highest peak three days after urine application. As expected, the highest N_2O peak was from $S_{r/w}\_U_{r/w}$, with a peak emission of 0.7 ± 0.1 kg N ha^{-1} day^{-1} on day 3 after urine application.

Overall, there was a sward treatment effect (P= 0.02) on the cumulative N_2O emissions (Table 5.3). The cumulative N_2O emissions in the plantain swards was 50% lower (9 ± 2.7 kg N ha^{-1}) compared to the ryegrass-white clover (18 ± 3.6 kg N ha^{-1}) swards. Aucubin reduced (P= 0.05) N_2O emissions in ryegrass-white clover sward but not in plantain sward (Table 5.4). In both sward treatments, N_2O emissions from urine treatments reached background levels on day 65 after treatment application. Plantain sward tended to decrease (*P*= 0.08) the EF_3 compared to the EF_3 from ryegrass-white clover swards.

When urine from cows fed either plantain or ryegrass-white clover was diluted to the same N concentration and applied onto a ryegrass-white clover sward, there was no urine effect (*P*= 0.93) (Table 5.5) on the cumulative N_2O emissions (Figure 5.3c). The ryegrass-white clover_diluted urine exhibited a major peak on day 3 after urine application. Daily N_2O emissions were similar from both ryegrass-white clover_diluted and plantain urines and they reached background levels on day 56 after treatments applications.

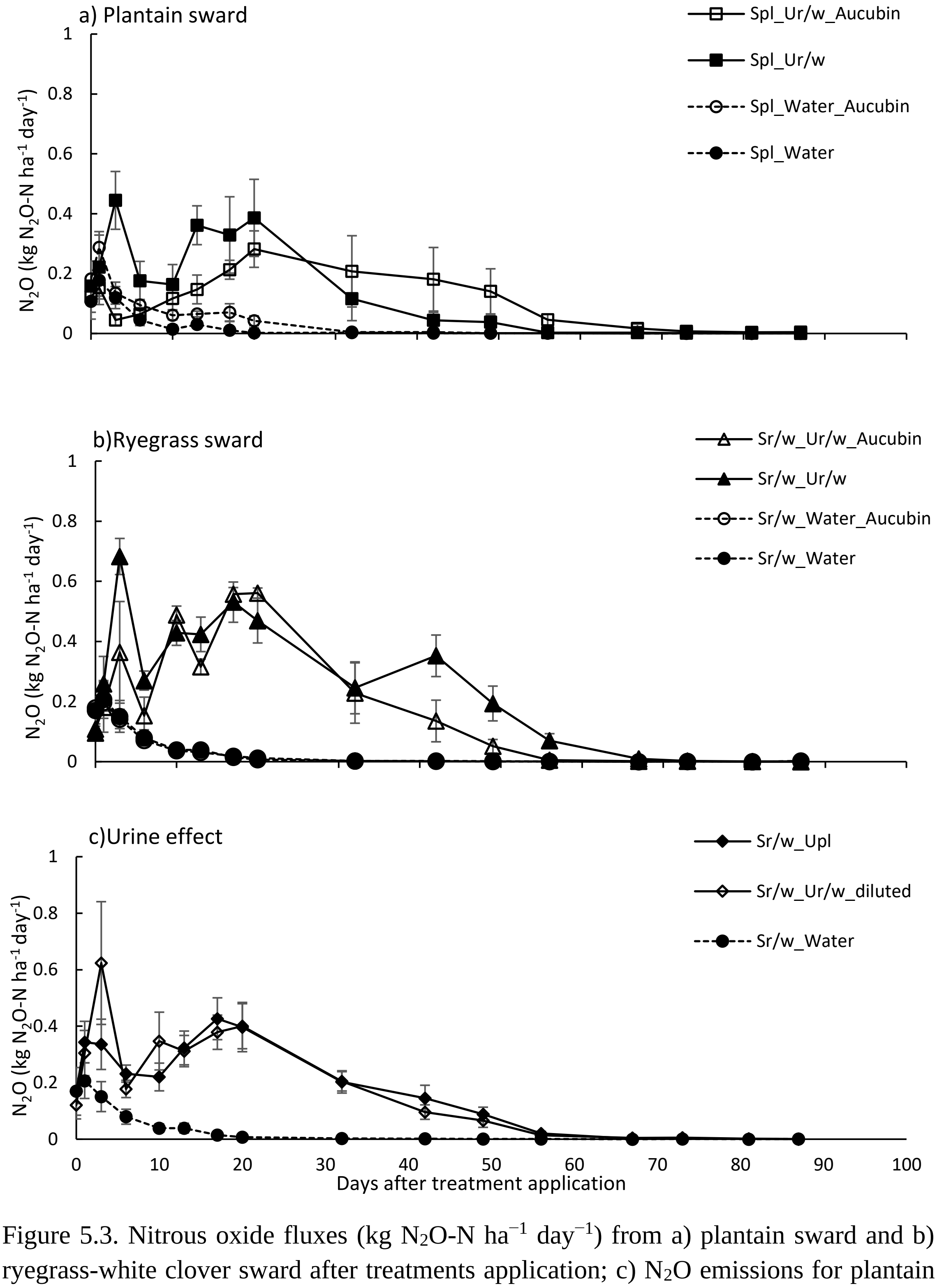


Figure 5.3. Nitrous oxide fluxes (kg N_2O-N ha^{-1} day^{-1}) from a) plantain sward and b) ryegrass-white clover sward after treatments application; c) N_2O emissions for plantain urine and ryegrass-white clover urine diluted to the same N concentration as plantain urine, applied to ryegrass-white clover swards; a control was also included where water was applied. Error bars are SEM (n=5). S_{pl} = plantain sward, $S_{r/w}$ = ryegrass-white clover sward, U_{pl} = plantain urine, $U_{r/w}$ = ryegrass-white clover urine; $U_{r/w\_diluted}$ = ryegrass-white clover urine diluted to the same N concentration that plantain urine.

Table 5.3. Cumulative N_2O-N emissions (kg N_2O-N ha^{-1}), and emission factors (EF_3%) for plantain and ryegrass-white clover swards after ryegrass-white clover urine application.

Swards	**Cumulative N_2O-N (kg N ha^{-1})**	**EF_3**
Ryegrass-white clover	18 ± 3.6^{a}	3.3 ± 0.9
Plantain	9 ± 2.7^{b}	1.2 ± 0.5
Significance		
Sward	0.02	0.08

Values are mean ± SEM (n=5). ($P < 0.05$).

Table 5.4. Aucubin effect on the cumulative N_2O-N emissions (kg N_2O-N ha^{-1}) for plantain and ryegrass-white clover swards after aucubin application.

	Cumulative N_2O-N (kg N ha^{-1})		
Swards	Without aucubin	Aucubin	**Significance** (Sward(aucubin))
Plantain	11 ± 3.5^{a}	8 ± 2.7^{a}	0.40
Ryegrass-white clover	22 ± 2.3^{a}	14 ± 2.1^{b}	0.05

Values are mean ± SEM (n=5). ($P < 0.05$).

Table 5.5. Cumulative N_2O-N (kg N ha^{-1}) emissions and EF_3, cumulative NO_3^--N (kg N ha^{-1}) and total N (kg N ha^{-1}) leaching for urine of cows fed plantain and urine from cows fed ryegrass-white clover diluted.

	Cumulative N_2O-N (kg N ha^{-1})	**EF_3 (%)**	**Cumulative NO_3^--N (kg N ha^{-1})**	**Total N (kg N ha^{-1})**
$S_{r/w}$_$U_{r/w_diluted}$	13 ± 2	2.5 ± 0.5	79 ± 14	91 ± 6
$S_{r/w}$_ U_{pl}	13 ± 2	2.6 ± 0.5	64 ± 15	105±12
Significance				
Urine	0.93	0.73	0.47	0.34

Values are mean ± SEM (n=5); EF_3: emission factor

5.4.2.3 Nitrate leaching and total N

Urine application to the lysimeters had higher ($P< 0.0001$) NO_3^- and total N leaching concentrations compared to lysimeters that only received water (Table 5.6). The peak of NO_3^- leaching (Figure 5.4) and total N leaching (Figure 5.5) occurred 27 and 19 days after treatments application in the plantain and ryegrass-white clover swards, respectively. Cumulative NO_3^- leaching from plantain and ryegrass-white clover swards with urine were 63 and 82 kg NO_3^--N ha^{-1}, respectively. In plantain and ryegrass-white clover swards with the application of aucubin the cumulative NO_3^- leaching was 59 and 54 kg NO_3^--N ha^{-1}, respectively. The effects of the sward (P= 0.37) and aucubin (P= 0.56) treatments on NO_3^- leaching were not significant (Table 5.6).

Cumulative total N leaching losses from the plantain sward and ryegrass-white clover sward for the water treatment were 14 and 7 kg N ha^{-1}, respectively, and for the urine treatment were 105 and 106 kg N ha^{-1}, respectively. While there was a general trend of treatments receiving aucubin having lower N losses in drainage, this was not statistically significant. The effects of the sward (P= 0.80) and aucubin (P= 0.69) treatments on total N leaching were not significant (Table 5.6). Cumulative NO_3^- and total N leaching under diluted ryegrass-white clover and plantain urine, applied to ryegrass-white clover sward, were not statistically different (Table 5.5) (P= 0.47 and P= 0.34, respectively).

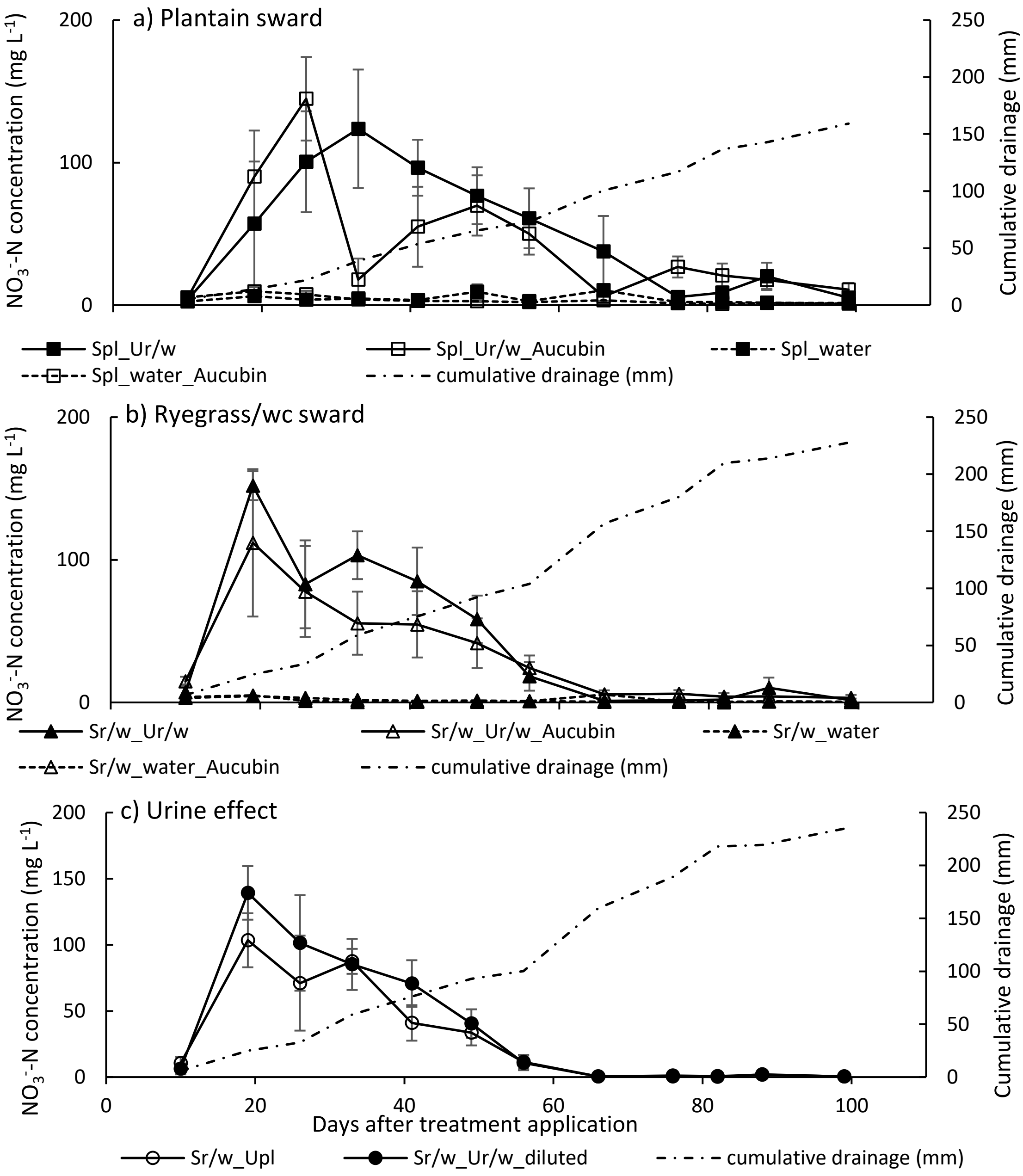


Figure 5.4. Nitrate leaching (mg L^{-1}) from a) plantain sward and b) ryegrass-white clover sward after treatments application; c) Nitrate leaching for plantain urine and ryegrass-white clover_diluted to the same N concentration that plantain urine, applied to ryegrass-white clover swards; a control was also included were water was applied. Error bars are SEM (n=5). Refer to Figure 5.3 for treatment names.

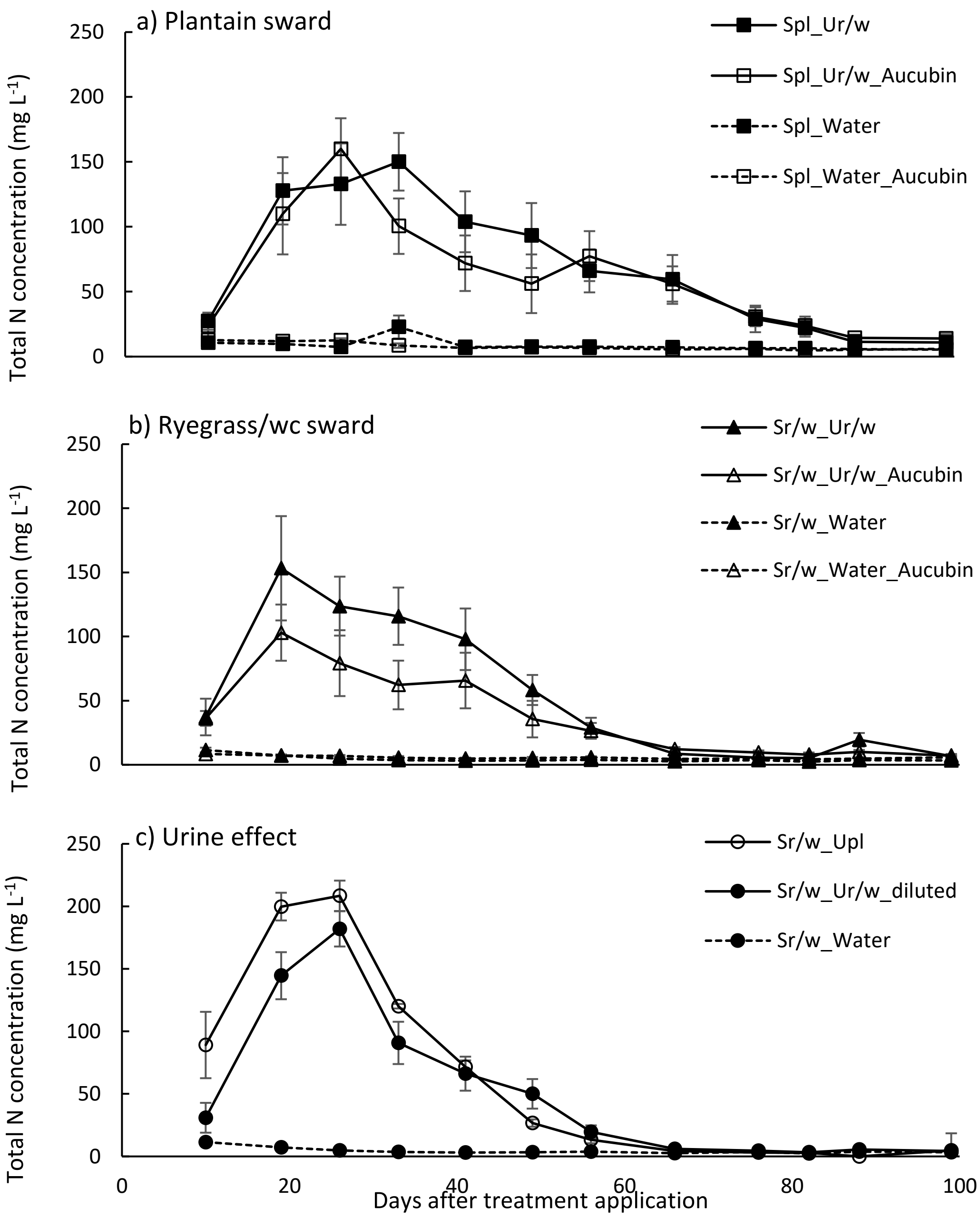


Figure 5.5. Total N (mg L^{-1}) from plantain sward (a) and ryegrass-white clover sward (b) after treatments application; c) Total N leaching for plantain urine and ryegrass-white clover_diluted to the same N concentration that plantain urine, applied to ryegrass-white clover swards; a control was also included were water was applied. Error bars are SEM (n=5). Refer to Figure 5.3 for treatment names.

Table 5.6. Cumulative NO_3^- -N (kg N ha^{-1}) leaching, and total N leached (kg N ha^{-1}) for the two sward treatments (plantain and ryegrass-white clover) with and without aucubin application. (Values are mean ± SEM)

	Cumulative NO_3^--N (kg N ha^{-1})	**Cumulative total N (kg N ha^{-1})**
$S_{r/w}$_$U_{r/w}$	82 ± 17	105 ± 29
$S_{r/w}$_$U_{r/w}$ _Aucubin	54 ± 29	64 ± 32
$S_{r/w}$_Water	11 ± 6	14 ± 0.8
$S_{r/w}$_Water _Aucubin	5 ± 1	8 ± 0.8
S_{pl}_$U_{r/w}$	63 ± 29	106 ± 47
S_{pl}_$U_{r/w}$_Aucubin	59 ± 18	94 ± 28
S_{pl}_Water	3 ± 0.6	7 ± 2
S_{pl}_Water _Aucubin	3 ± 0.5	12 ± 1
Significance		
Aucubin	0.37	0.69
Sward	0.56	0.80
Aucubin*sward	0.78	0.64

The cumulative drainage volume for plantain and ryegrass-white clover swards were 159 and 228 mm, respectively, which were not significantly different.

5.4.2.4 Nitrogen uptake and dry matter yield

In both the plantain and the ryegrass-white clover swards, urine application increased ($P<0.001$) the N uptake and dry matter yield compared to water application. The aucubin application in both sward treatments did not have any effect on N uptake by plants ($P=$ 0.57) nor the dry matter yield from the swards (Table 5.6). The dry matter yield was greater (P = 0.03) in ryegrass-white clover swards than in plantain swards (Table 5.6).

Table 5.7. Nitrogen uptake (kg N ha^{-1}) by the swards and dry matter yield (kg DM ha^{-1}) at the end of the experimental period (n=5).

Treatments	**N uptake (kg N ha^{-1})**	**Dry matter (kg ha^{-1})**
$S_{r/w}$ _$U_{r/w}$	152 ± 21	3514 ± 296
$S_{r/w}$_$U_{r/w}$ _Aucubin	112 ± 39	3586 ±708
$S_{r/w}$_Water	61 ± 5	2569 ±83
$S_{r/w}$_Water _Aucubin	65 ± 7	2579 ±194
S_{pl} _$U_{r/w}$	92 ± 25	2733 ±326
S_{pl} _ $U_{r/w}$ _Aucubin	123 ± 37	3103 ±491
S_{pl}_Water	64 ± 11	2465 ±108
S_{pl}_Water_Aucubin	94 ± 8	2659 ±154
Significance		
Aucubin	0.6	0.9
Sward	0.6	0.03
Aucubin*sward	0.4	0.6

Values are mean ± SEM (n=5); in the statistical analysis the treatments with water were not included.

5.5 DISCUSSION

The current experiment suggests that the plantain sward and aucubin reduce the N_2O emissions from urine patches, despite no significant effect being observed on NO_3^- leaching. The reduction in N_2O emissions from urine patches in the plantain sward was consistent with results from other experiments where N_2O emissions from plantain sward were lower than from ryegrass-white clover (Luo *et al.,* 2018; Rodriguez *et al.*, 2020; Simon *et al.*, 2019). Pijlman *et al.* (2019) reported that the presence of plantain reduced N_2O fluxes by 39% compared to perennial ryegrass, which is similar to the reduction in N_2O emissions found in the current lysimeter experiment.

In the current study, the lower N_2O emissions from the plantain sward could be attributed to the presence of the natural aucubin already released by the plantain sward directly into the soil. Research has suggested that the reduction in N_2O emissions from plantain swards could be due to the presence of secondary metabolites produced by plantain inhibiting the nitrification process in soils (de Klein *et al.*, 2019; Dietz *et al.*, 2013; Gardiner *et al.*, 2017; Luo *et al.*, 2018; Simon *et al.*, 2019). Plantain produces the secondary metabolites catalpol and aucubin (Stewart, 1996; Tamura & Nishibe, 2002). These secondary metabolites can be found in the leaves, reproductive stem and roots of plantain (Darrow & Bowers 1999). Recently, de Klein *et al.* (2019) hypothesised that plantain swards could affect N_2O emissions by the release of the secondary metabolites/iridoid glycoside, aucubin and catalpol in root exudates and that these would inhibit the nitrification process. In the hydroponic experiment reported here, only catalpol was detected but not aucubin in the root exudates of plantain. Both aucubin and catalpol were found in plantain's leaves throughout the experiment. Very low concentrations of catalpol were found in both Tonic and Agritonic cultivars of plantain ranging from 0.1 g kg^{-1} DM in Tonic to 0.5 g kg^{-1} DM in Agritonic. This result contrasts with previous results, where Tonic plantain did not produce catalpol (Al-Mamun *et al.*, 2008; Navarrete *et al.*, 2016; Stewart, 1996; Tamura & Nishibe, 2002). The catalpol and aucubin concentrations in the leaves are affected by the leaf age (Tamura & Nishibe, 2002). Catalpol and aucubin may be excreted in the urine of cows grazing plantain swards and this could potentially be another input of these secondary metabolites to the soil (Judson *et al.*, 2019).

Although in the current experiment aucubin concentration in plantain roots was not determined, this could be another source of aucubin into the soil when roots die and the material is decomposed in the soil. We only considered the aucubin concentration of leaves but it is possible that the aucubin concentration in the roots was similar to that in the leaves (Miehe-Steier *et al*., 2015). Once the leaf material senescence, it is returned to the soil and could be incorporated through animal trampling.

The glasshouse conditions under which plantain was grown in the current hydroponic experiment could be another reason for the results observed in the root exudates. In damaged plantain plants, catalpol concentration in leaves were higher compared to undamaged plants (Bowers *et al.*, 1993). Darrow *et al*. (1999) found that the concentration of catalpol and aucubin were higher in reproductive stems than in the roots. They also reported that iridoid glycoside concentrations were higher in roots grown in low nutrient concentrations compared to high nutrient conditions. More recently, Wurst *et al*. (2010) reported that when plantain was exposed to soil micro-organisms, the aucubin concentration of the roots increased without affecting the concentration in the rhizosphere. Interestingly, the presence of nematodes increased the concentration of aucubin and catalpol in the root exudates, but it did not affect the concentration in the roots. However, it is not clear if this is a plant defence mechanism or by-product of the feeding nematodes. In the current experiment, the nutrient solution was changed weekly to ensure good nutrient status, which may have influenced lower excretion of those compounds. Moreover, due to it being a hydroponic experiment the plants were not exposed to any type of soil micro-organisms or nematodes, and that could also explain the low release of the secondary metabolites.

Aucubin decreased the cumulative N_2O emissions in ryegrass-white clover sward. Gardiner *et al*. (2019a) reported the results from a field experiment, where cow urine (700 kg N ha^{-1}) was mixed with either a plantain leaf extract (incorporating all the secondary metabolites into the soil) or with commercial aucubin (47 kg ha^{-1}). They found that N_2O emission factors (EF) were 50% and 70% lower than for the urine only treatment, respectively. In a laboratory study (Dietz *et al*., 2013), where 12 mg of undissolved aucubin was applied to the soil at a rate of 0.60 mg g^{-1} soil dry weight, soil concentrations of NO_3^- were 46% lower and NH_4^+ were 31% higher compared to the no aucubin control during the first 28 days of the incubation, which supports the notion that aucubin suppresses nitrification.

The results of NO_3^- and total N leaching from plantain swards from this experiment were in contrast with others lysimeter studies that observed a reduction in NO_3^- leaching when urine was applied to pasture containing plantain compared to ryegrass-white clover swards (Carlton *et al*., 2018; Welten *et al*., 2019; Woods *et al*., 2018). In the current study, there was a delay in the peak of NO_3^- leaching from the plantain sward compared to the ryegrass-white clover sward, however, there were no differences in the cumulative NO_3^- leaching. The cumulative drainage volume was lower under the plantain sward (159 mm) than the ryegrass-white clover sward (228 mm), but the difference was not significant. The lack of differences in NO_3^- leaching between treatments were probably due to the high variability of the drainage from the soil. Reductions in NO_3^- leaching from previous experiments from plantain sward ranged between 39 and 78%. Welten *et al*. (2019) observed that the efficacy of plantain in reducing NO_3^- leaching increased from 15 to 50% from summer urine application to winter urine application compared to ryegrass. This reduction was attributed to a lower drainage volume coupled with a low N concentration in the drainage water from plantain compared to ryegrass swards. The other reason for the efficacy of plantain in reducing NO_3^- leaching compared to ryegrass is the presence of the secondary metabolites in plantain. Carlton *et al*. (2018) quantified the abundance of ammonia oxidising bacteria (AOB) which contribute to the nitrification process in high soil N environments (Di *et al*. 2009). Carlton et al. (2018) observed that the growth rate of AOB was inhibited with the presence of plantain in the sward, reducing the rate of the soil nitrification. Although previous studies did not quantify the concentration of aucubin/catalpol in the roots, in our hydroponic experiment we only found catalpol in the root exudates of plantain. Aucubin can probably be incorporated via leaf litter.

A lack of effect on N_2O emissions and NO_3^- leaching when urine from cows grazing ryegrass-white clover or plantain was applied at the same N loading rate to the ryegrass-white clover sward suggests that there were no secondary metabolites in the plantain urine. During autumn 2019, the plantain sward contained 28% of plantain DM (Chapter 3), which could be the reason for the lack of the effect of the urine of cows fed on plantain pastures. Similarly, Simon *et al.* (2019) applied urine from cows fed 45% plantain (urine N rate of 560 kg N ha^{-1}) and 0% plantain diluted to the same N concentration as 45%

plantain, and did not find any difference in cumulative N_2O emissions. In contrast, Judson *et al.* (2019) observed that applying urine from cows fed pure plantain and urine from cows fed ryegrass-white clover, with similar total N concentrations, resulted in lower nitrification from the urine of cows grazing plantain after the first 14 days following application. Soil NH_4^+ concentrations also confirmed their results, with the ryegrass-white clover urine treatment showing lower NH_4^+ concentrations compared to the plantain urine treatment, indicating that plantain urine inhibited nitrification. According to these results, BNI compounds could be excreted in the urine of cows fed plantain swards, increasing the nitrification inhibitor potential of the plantain sward. However, further research is needed to determine if these secondary metabolites are excreted in the urine and are present in the soil.

5.6 CONCLUSIONS

The plantain sward and aucubin decreased N_2O emissions compared to the ryegrass-white clover sward likely by inhibiting the soil nitrification process. Catalpol but not aucubin, was detected in the root exudates of plantain. Both catalpol and aucubin were found in the plantain’s leaves. In this experiment, aucubin was demonstrated to be responsible for the decrease in N_2O emissions, probably via leaf litter. Urine from cows fed plantain did not reduce N_2O emissions relative to urine from cows grazing ryegrass-white clover pastures, when applied at the same N urine rate, suggesting there was no biological nitrification inhibitors in the urine. Nitrate leaching was not affected by the aucubin, sward or urine treatments but this is likely to be attributable to the high variability between the lysimeters, and that at the bottom of the lysimeters the clay layer could have affected the drainage.

5.7 REFERENCES

Al-Mamun, M., Abe, D., Kofujita, H., Tamura, Y. & Sano, H. (2008). Comparison of the bioactive components of the ecotypes and cultivars of plantain (Plantago lanceolata L.) herbs. *Animal Science Journal, 79*(1), 83-88.

Bartholomaeus, A. & Ahokas, J. (1995). Inhibition of P-450 by aucubin: is the biological activity of aucubin due to its glutaraldehyde-like aglycone? *Toxicology Letters, 80*(1), 75-83.

Blakemore, L. C. Methods for chemical analysis of soils. (1987). *NZ Soil Bur Sci Rep., 80*,71–76.

Bowers, M. D. & Stamp, N. E. (1993). Effects of Plant Age, Genotype and Herbivory on Plantago Performance and Chemistry. *Ecology, 74*(6), 1778-1791.

Box, L. A., Edwards, G. R. & Bryant, R. H. (2017). Milk production and urinary nitrogen excretion of dairy cows grazing plantain in early and late lactation. *New Zealand Journal of Agricultural Research, 60*(4), 470-482.

Box, L. A. & Judson, H. G. (2018). The concentration of bioactive compounds in Plantago lanceolata is genotype specific. *Journal of New Zealand Grassland, 80*, 113- 118.

Cameron, K. C., Di, H. J. & Moir, J. L. (2013). Nitrogen losses from the soil/plant system: a review. *Annals of Applied Biology, 162*(2), 145-173.

Carlton, A. J., Cameron, K. C., Di, H. J., Edwards, G. R. & Clough, T. J. (2018). Nitrate leaching losses are lower from ryegrass/white clover forages containing plantain than from ryegrass/white clover forages under different irrigation. *New Zealand Journal of Agricultural Research*, *62,* 150-172.

Darrow, K. & Bowers, M. D. (1999). Effects of Herbivore Damage and Nutrient Level on Induction of Iridoid Glycosides in Plantago lanceolata. *Journal of Chemical Ecology, 25*(6), 1427-1440.

Davini, E., Javarone, C., Trogolo, C., Aureli, P. & Pasolini, B. (1986). The quantitative isolation and antimicrobial activity of the aglycone of aucubin. *Phytochemistry, 25*(10), 2420-2422.

de Klein, C. A. M., Barton, L., Sherlock, R. R., Li, Z. & Littlejohn, R. P. (2003). Estimating a nitrous oxide emission factor for animal urine from some New Zealand pastoral soils. *Soil Research*, *41*(3), 381-399.

de Klein, C. A. M., van der Weerden, T. J., Luo, J., Cameron, K. C. & Di, H. J. (2019). A review of plant options for mitigating nitrous oxide emissions from pasture-based systems. *New Zealand Journal of Agricultural Research*, *63*(1), 29-43.

Di, H. J. & Cameron, K. C. (2002a). Nitrate leaching in temperate agroecosystems: sources, factors and mitigating strategies. *Nutrient Cycling in Agroecosystems, 64*(3), 237-256.

Di, H. J. & Cameron, K. C. (2002b). 'The use of a nitrification inhibitor, dicyandiamide (DCD), to decrease nitrate leaching and nitrous oxide emissions in a simulated grazed and irrigated grassland', *Soil Use and Management*, *18,* 395-403.

Di, H. J. & Cameron, K. C. (2012). How does the application of different nitrification inhibitors affect nitrous oxide emissions and nitrate leaching from cow urine in grazed pastures? *Soil Use and Management, 28*(1), 54-61.

Di, H. J., Cameron, K. C., Shen, J. P., Winefield, C. S., O'callaghan, M., Bowatte, S. & He, J. Z. (2009). Nitrification driven by bacteria and not archaea in nitrogen-rich grassland soils. *Nature Geoscience, 2*(9), 621-624.

Dietz, M., Machill, S., Hoffmann, H. C. & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil, 368*(1-2), 445-458.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019a). Efficacy of aucubin as a nitrification inhibitor assessed in two Canterbury field trials. *New Zealand Journal of Agricultural Research*, *63*(1),73-86.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J. & Edwards, G. R. (2019b). Ruminant urine patch nitrification and N_2O flux: effects of urine aucubin rate in a laboratory trial. *New Zealand Journal of Agricultural Research*, *63*(1), 65-72.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J., Edwards, G. R. & de Klein, C. A. M. (2017). Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. *New Zealand Journal of Agricultural Research*, *61* (4) 495-503.

Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R. & de Klein, C.A.M. (2016). Potential for forage diet manipulation in New Zealand pasture ecosystems to mitigate ruminant urine derived N_2O emissions: a review. *New Zealand Journal of Agricultural Research*, *59*(3), 301-317.

Haynes, R. J. & Williams, P. H. (1993). Nutrient cycling and soil fertility in the grazed pasture ecosystem. *Advances in agronomy, 49*, 119-199.

Hosomi, M. & Sudo, R. (1986). Simultaneous determination of total nitrogen and total phosphorus in freshwater samples using persulfate digestion. *International Journal of Environmental Studies, 27*(3-4), 267-275.

Judson, H. G., Fraser, P. M. & Peterson, M. E. (2019). Nitrification inhibition by urine from cattle consuming *Plantago lanceolata*. *Journal of New Zealand Grasslands, 81*, 111-116.

Ledgard, S., Schils, R., Erikson, J. & Luo, J. (2009). Environmental impacts of grazed clover/grass pastures. In: Teagasc, Oak Park, Carlow, Ireland.

Luo, J., Balvert, S. F., Wise, B., Welten, B., Ledgard, S. F., de Klein, C. A. M., Lindsey, S. & Judge, A. (2018). Using alternative forage species to reduce emissions of the greenhouse gas nitrous oxide from cattle urine deposited onto soil. *Science of The Total Environment, 610–611*, 1271-1280.

Luo, J., Hoogendoorn, C., van der Weerden, T., Saggar, S., de Klein, C., Giltrap, D., Rollo, M. & Rys, G. (2013). Nitrous oxide emissions from grazed hill land in New Zealand. *Agriculture, Ecosystems & Environment, 181*, 58-68.

Miehe-Steier A., Roscher C., Reichelt M., Gershenzon J., Unsicker S. B. (2015). Light and nutrient dependent responses in secondary metabolites of *Plantago lanceolata* offspring are due to phenotypic plasticity in experimental grasslands. *PLoS ONE 10*(9): e0136073.

Minnée, E. M. K., Leach, C. M. T. & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science, 239*, 104093.

Moir, J. L., Cameron, K. C., Di, H. J. & Fertsak, U. (2011). The spatial coverage of dairy cattle urine patches in an intensively grazed pasture system. *The Journal of Agricultural Science, 149*(4), 473-485.

Navarrete, S., Kemp, P. D., Pain, S. J. & Back, P. J. (2016). Bioactive compounds, aucubin and acteoside, in plantain (*Plantago lanceolata L.*) and their in vitro rumen fermentation. *Animal Feed Science and Technology, 222*, 158-167.

Pijlman, J., Berger, S. J., Lexmond, F., Bloem, J., van Groenigen, J. W., Visser, E. J. W., Erisman., J. W. & van Eekeren, N. (2019). Can the presence of plantain (Plantago lanceolata L.) improve nitrogen cycling of dairy grassland systems on peat soils? *New Zealand Journal of Agricultural Research,* 63(1), 106-122.

Rodriguez, M. J., Kemp, P. D., Navarrete, S., Hanly, J. A., Horne, D. J. & Bishop, P. (2020). Nitrogen losses from plantain: what can we say? In: Nutrient Management in Farmed Landscapes. (Eds. C.L. Christensen, D.J. Horne and R. Singh). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 33. Farmed Landscapes Research Centre, Massey University, Palmerston North, New Zealand. 8 pages.

Selbie, D. R., Buckthought, L. E. & Shepherd, M. A. (2015). The Challenge of the Urine Patch for Managing Nitrogen in Grazed Pasture Systems. *Advances in agronomy, 129*, 229-292.

Simon, P. L., de Klein, C. A. M., Worth, W., Rutherford, A. J. & Dieckow, J. (2019). The efficacy of Plantago lanceolata for mitigating nitrous oxide emissions from cattle urine patches. *Science of The Total Environment*, *691*, 430-441.

Stark, C. & Richards. K. G. (2008). The continuing challenge of agricultural nitrogen loss to the environment in the context of global change and advancing research. *Dynamic Soil, Dynamic Plant, 2*, 1-12.

Stewart, A. V. (1996). Plantain (Plantago lanceolata)—a potential pasture species. Proc. N. Z. Grassland Assoc. *58*, 77–86.

Subbarao, G. V., Wang, H. Y., Ito, O., Nakahara, K. & Berry, W. L. (2007). NH_4^+ triggers the synthesis and release of biological nitrification inhibition compounds in *Brachiaria humidicola* roots. *Plant and Soil, 290*(1), 245-257.

Tamura, Y. & Nishibe, S. (2002). Changes in the Concentrations of Bioactive Compounds in Plantain Leaves. *Journal of Agricultural and Food Chemistry, 50*(9), 2514-2518.

van der Weerden, T. J., Clough, T. J. & Styles, T. M. (2013). Using near-continuous measurements of N2O emission from urine-affected soil to guide manual gas sampling regimes. *New Zealand Journal of Agricultural Research, 56*(1), 60-76.

Welten, B. G., Ledgard, S. F., Judge, A. A., Sprosen, M. S., McGowan A. W. & Dexter, M. M. (2019). Efficacy of different temperate pasture species to reduce nitrogen leaching from cattle urine applied in different seasons: A soil lysimeter study. *Soil Use and Management, 35*(4), 653-663.

Woods, R. R., Cameron, K. C., Edwards, G. R., Di, H. J., & Clough, T. J. (2018). Reducing nitrogen leaching losses in grazed dairy systems using an Italian ryegrass-plantain-white clover forage mix. *Grass and Forage Science, 73*(4), 878-887.

Wurst, S., Wagenaar, R., Biere, A. & van der Putten, W. H. (2010). Microorganisms and nematodes increase levels of secondary metabolites in roots and root exudates of Plantago lanceolata. *Plant and Soil, 329*(1), 117-126.

6 GENERAL DISCUSSION AND CONCLUSIONS

6.1 OBJECTIVE OF THE THESIS

This thesis reports experiments conducted in the field, a lysimeter experiment and a hydroponic experiment to determine the effectiveness of using plantain as a strategy to reduce NO_3^- leaching, and N_2O and NH_3 emissions from dairy systems. The results from the experiments were presented and discussed in detail in Chapters 3, 4 and 5. In brief, Chapter 3 reports on a field study that evaluated the milk production, urine N and urea-N concentrations of cows, and NO_3^- leaching from plantain, plantain-clovers mix, and ryegrass-white clover mix swards treatments during two full grazing seasons. The field experiment in Chapter 3 is the first study quantifying the effects of grazed plantain swards on NO_3^- leaching at a large plot scale. Chapter 4 compared the N_2O and NH_3 emissions from patches of urine from cows fed plantain with emissions from urine from cows fed ryegrass-white clover swards. Chapter 5 evaluated the effect of aucubin application on NO_3^- leaching and N_2O emissions and studied the ability of the root systems of plantain to release the secondary metabolites responsible for the inhibition of the nitrification. This general discussion chapter presents an integrated discussion of the key findings from the experimental chapters.

6.2 MAIN FINDINGS

In this thesis, it was observed that the use of plantain is a mitigation strategy capable of reducing NO_3^- leaching, N_2O and NH_3 emissions losses compared with ryegrass-white clover swards. The reductions in N losses achieved by plantain swards occurred due to treatment effects in both the animal and in the soil, however, it was beyond the scope of this thesis to study the details of the mechanisms responsible for these reductions.

In intensive dairy systems, the ingested N that is not utilised by the grazing animals is redistributed through excreta deposition. Studies have found that NO_3^- from cows' urine that has been deposited during grazings in summer, autumn and early winter period has the greatest effect on the amount of NO_3^- that leaches during winter and early spring (Christensen *et al.*, 2018; Shepherd *et al.*, 2011, 2017). Nitrogen excreted in the urine of cows is the major source of NO_3^- leaching from grazed pastures, and reducing the N concentration in urine and the total N loading into the soil would lead to a reduction in NO_3^- leaching from pastoral systems (Ledgard *et al.*, 2015).

During the summer and autumn of both grazing seasons of the current study (Chapter 3), N intake was similar for cows fed pure plantain and ryegrass-white clover pastures. Nitrogen intake by cows grazing the plantain-clovers mix in the summer and autumn of 2018 was much greater than from the other two treatments. Cow urine N and urea-N concentrations were lower for the sward containing the plantain and plantain-clovers mix during the 2018 drainage season. A reduction in urinary N concentration directly affects N losses from urine patches. In the 2018 drainage season, the pure plantain swards reduced NO_3^- leaching losses by 48 and 58% (2.6 and 3.8 kg NO_3^- ha^{-1}) in comparison to ryegrass-white clover and plantain-clovers mix swards, respectively. This decrease was likely due to an effect of plantain in the cows. From the results of this experiment, it is not conclusive that the reduction in NO_3^- leaching was due to a plantain soil effect. The NO_3^- leaching between ryegrass-white clover and plantain-clovers mix pastures was not different during the first drainage season, however, the total apparent N intake was greater in plantain-clover mix pastures. If a plantain soil effect would take place, a reduction in NO_3^- leaching from plantain-clovers mix would be expected.

These reductions in UNc and urea-N concentrations were achieved during summer to early winter, which is the ‘critical period’ when NO_3^- accumulates in the soil profile and it is prone to leaching in the following drainage season. During that period in 2018, the plantain content in the pastures was greater than 30%, which is commonly acknowledged as the minimum content required for appreciable reductions in N leaching (Minnée *et al.*, 2020). During the 2019 drainage season, the NO_3^- leaching losses were similar between pure plantain and ryegrass-white clover swards, and greater for plantain-clovers mix swards. During the critical period of this grazing season, the plantain content of both swards was less than 30%. Furthermore, the clover content of the plantain-clovers mix and pure plantain swards ranged from 34 to 36% and 17 to 22%, respectively, during the summer/autumn of 2019 and may have influenced NO_3^- leaching losses from these plots. This supports the notion that there might be a lower limit to the percentage of plantain required in a mixed sward to reduce NO_3^- leaching and that the clover content may also be an important consideration.

In the field study described in Chapter 4, a plantain sward (soil) effect was observed in the spring of 2017. Nitrous oxide losses from urine from cows fed plantain and ryegrass-white clover pastures were 31% (1.4 kg N_2O ha^{-1}) lower from the plantain sward than from the ryegrass-white clover swards (Chapter 4). The plantain sward effect was most likely related to the release of secondary metabolites/iridoid glycosides, which can act as biological nitrification inhibitors (Dietz *et al*., 2013; Gardiner *et al*., 2017). However, at this time, there was no urine effect on N_2O emissions (Chapter 4); N_2O losses from urine of cows fed pure plantain were similar to emissions from the urine of cows fed ryegrass-white clover swards. In the autumn/winter of 2018, N_2O emissions from urine of cows fed pure plantain sward were 49% lower (4.4 kg N_2O ha^{-1}) than urine of cows fed ryegrass-white clover sward. Unexpectedly and in contrast to the 2017 spring results, the emissions were 69% (7.1 kg N_2O ha^{-1}) higher from plantain swards and this was most likely due to the influence of soil moisture content. In autumn/winter, the water filled pore space (WFPS) was greater in plantain soils which is likely to have increased the N_2O emissions and the emission factor from plantain sward compared to ryegrass-white clover swards. It is also possible that NO_3^- concentrations were greater under the plantain sward than the ryegrass-white clover pasture due to the relatively slow growth rate of plantain at this time of the year.

This result highlights the need to consider not only the presence of plantain in a sward but also other soil and environmental conditions which affect N_2O emissions. Although no effect of plantain urine was observed on N_2O emissions in spring 2017, in both spring 2017 and autumn/winter 2018, NH_3 losses from urine of cows fed plantain were 80 and 90% (10.2 and 7.4 kg NH_3 ha^{-1}) lower than urine of cows fed ryegrass-white clover, respectively, due to urea concentration in urine being half those of urine from cows fed ryegrass-white clover swards.

In the hydroponic experiment (Chapter 5), only catalpol was detected in the plantain root system after 50 and 60 days of transplantation (Chapter 5). Both aucubin and catalpol were detected in the leaves of plantain species. When aucubin was applied to ryegrass-white clover swards, N_2O emissions were reduced by 36% (6 kg N_2O ha^{-1}). A plantain sward effect was also detected on N_2O emissions; the plantain sward reduced N_2O emissions by 50% (9 kg N_2O ha^{-1}) compared to ryegrass-white clover swards. There was no observed difference in NO_3^- leaching and N_2O emissions when urine from cows fed plantain and urine from cows fed ryegrass-white clover, both with the same total N concentration, were applied to lysimeters containing ryegrass-white clover swards. Taken in isolation, this result suggests that little aucubin is added to the soil in urine. Form this

Chapter it can be concluded that different factors increased the N_2O emissions, and in these lysimeters, the high N_2O emissions could be attributted to high denitrification rates due to the fine texture and poorly drained nature of the Tokomaru silt loam. The results from Chapter 5 also indicate that the plantain sward effect is attributed to aucubin released into the soil from plantain plants, probably via leaf litter.

The field experiments carried out in Chapter 3 and 4 generated data at the plot scale and urine patch level, respectively. To extend this consideration of the advantages of plantain to the farm scale, the effects of plantain were simulated using OVERSEER Ed, a nutrient budgeting model. Results are shown in Table 6.1. The dairy farm used in this modelling exercise is based on Dairy Farm 4 at Massey University i.e. most of the required input parameters were sourced from Farm 4 information. Some modifications were carried out in this exercise and this farm consisted of mainly two blocks; the ‘effluent block’ (59 ha), and the ‘main block’ (141 ha). When plantain was adopted on the farm, the main block was divided so as to introduce an extra block where a sward containing plantain was grown, while maintaining the same total farm area. The plantain block represented 25 and 50% of the total area of the farm to determine how an increase in the area cultivated with plantain would affect N losses from a dairy system. In addition to the ‘control’ or base scenario, four scenarios with plantain pastures were modelled (Table 6.1). Two scenarios had a sward comprised of 30% of plantain and 70% of ryegrass-white clover. Two of the scenarios had a mixed pasture comprising 60% plantain and 40% ryegrass-white clover. In separate scenarios, the effect of different areas of the two mixed pastures was simulated. The mixed swards were assumed to cover 25% or 50% of the total farm area. In this way four scenarios were generated (two sward mixes x two areas of the mixed sward).

OVERSEER estimates that N leaching from the main block on the farm is 25 kg N ha^{-1} yr^{-1} and that the introduction of mixed swards of 30% and 60% plantain to this area would reduce leaching losses to 15 kg N ha^{-1} yr^{-1} and 11 kg N ha^{-1} yr^{-1}, respectively: this is a decrease of 40% and 56% in N leaching. These values agree reasonably well with the 48% decrease in N leaching measured for the plantain treatment in the field study (Chapter 3). When the whole-farm N leaching is considered for the four scenarios, the reduction in NO_3^- leaching losses ranges between 10 to 27% depending in the percentage of plantain in the pasture and the area that plantain covers in the farm (Table 6.1).

Table 6.1. Comparison of different scenarios modelled in OVERSEER Ed with increasing proportion of plantain in the cows' diet.

	N leaching (kg N ha^{-1} yr^{-1}) from the blocks						N_2O emissions	
Plantain (%) in the pasture	Plantain block	Main block	Effluent block	% of reduction*	N leaching from farm (kg N yr^{-1})	% of reduction at a farm level**	N_2O losses (eCO_2^{-1} $tonnes^{-1}$ yr^{-1})	% of reduction at a farm level**
0%		25	24	-	5163	-	352.6	-
30% (25% area of the farm)	15	25	24	40	4648	10	325.4	8
30% (50% area of the farm)	15	25	24	40	4138	20	298.2	15
60% (25% area of the farm)	11	25	24	56	4472	13	321.2	9
60% (50% area of the farm)	11	25	24	56	3787	27	289.8	18

* % of reduction in N leaching from plantain block compared to the main block; ** % of reduction in N leaching and N_20 losses from the whole farm.

For greenhouse gases data, OVERSEER only shows the results at a farm scale. In the spring 2017 experiment, where plantain sward reduced N_2O emissions by 31% compared to ryegrass-white clover sward, the N_2O emission loss from plantain swards was equivalent to 939 CO_2 equivalent kg^{-1} ha^{-1} yr^{-1} (3.15 kg N_2O ha^{-1} multiplied by 298 CO_2 equivalent kg^{-1} ha^{-1} yr^{-1}). In OVERSEER the reduction in N_2O emissions ranged from 8 to 18% when the area of the farm cultivated with plantain increased from 25 to 50% and when the proportion of plantain in the cows' diet increased from 30 to 60%, compared to a farm without plantain (Table 6.1). The OVERSEER model is underestimating the reduction in N_2O emission when a plantain sward is part of the system. One of the reasons for this result is that OVERSEER does not consider the effect of plantain on the soil, it only takes into account a reduction in the UNc of cows fed swards containing plantain.

The plantain- clovers mix swards increased the NO_3^- leaching during 2018-2019 grazing season and the main reason could be the higher clover content in the sward. However, in this sward there was not an inclusion of grass which could uptake more N and therefore, less N would be leached. Nowadays, there are farmers in the Tararua Plantain Project (Phillipa Hedley, DairyNZ, personal communication) who are managing plantain as a monoculture sward, and others who prefer to manage it in a mix. Therefore, there is no general rule to follow when grazing plantain and farmers are managing as is convenient for their farm. To see a beneficial effect of plantain on reducing urea and urine N concentration it is important to keep the percentage of plantain higher than 30%, which after two years of grazing is a challenge and farmers may need to re-sow. However, this is still under investigation.

This thesis highlights and quantifies the ability of plantain, as part of a dairy farm system, to reduce N losses to the aquatic and atmospheric environments. This study also highlights the importance of considering other factors that may affect the growth and persistence of plantain in the swards which may in turn impact on the environmental benefits of the sward. It is important to maintain a good growth and plant density in the plantain sward particularly going into autumn. Managing plantain in fine textured soils, with poor natural drainage, such as the Tokomaru silt loam may face unique challenges and it remain to be seen if plantain will perform as well in these soil types in the longer term.

6.3 FUTURE RESEARCH

From the three chapters of this thesis it was concluded that there was a plantain effect on the cows' urine and in the soil. However, a number of questions arise which require further study.

- Determine what proportions of the cow's diet should be made up of plantain if the environmental advantages of plantain are to be realised in a reliable and sustainable manner.

- Quantify the bioactive compounds concentration in plantain root exudates, and below and above-ground biomass. This may need to be based on plantain growing in soils so as to capture the effect of biotic stresses on the production of these compounds. This would enable an understanding of the role of the mechanism of the soil nitrification inhibition and the interaction with other factors such as season, soil type and management.

- Determine if aucubin or its derivates, aucubigenin, are present in the urine of cows fed on plantain. Although the results reported in Chapter 5 might suggest that secondary metabolites were not present in cow urine, a longer term and more detailed investigation may be required to confirm this result (e.g. multiple urine applications and/or urine from different seasons).

- Assess the effect of adding plantain shoot material to the soil and study the release of the bioactive compounds from this material to the soil.

- Assess the effect of plantain and its root system on the soil water content, soil pore size and water use efficiency. Soil moisture content is one of the main drivers for N_2O emissions and NO_3^- leaching therefore, it would be important to understand the changes caused by plantain in the soil microenvironment.

- Determine if the increased urine volume produced by cows grazing plantain is excreted in more, rather than larger, urination events, and determine if this results in larger areas covered with urine. If so, this may increase N_2O emissions and NO_3^- leaching and undermine some of the advantages reported at the patch level.

- Determine the long-term persistence of plantain and its performance, including its ability to mitigate N losses to the environment, in different soil types.

6.4 MAIN CONCLUSIONS

The research presented in this thesis has provided a comprehensive assessment of the use of plantain as a natural mitigation strategy to reduce N losses from a dairy system in comparison to ryegrass-white clover pastures.

This thesis demonstrated that:

- In comparison to ryegrass-white clover, a plantain sward reduced NO_3^- leaching losses from a grazed dairy system during the 2018 drainage season due to an effect at the cows' level i.e. N concentration in urine. Large reductions in urinary N and urea-N concentrations were achieved during late summer and autumn, where the plantain content in the pastures were greater than 30%, which directly affected the N outputs from the system. It would appear that if plantain comprises less than 30% of the sward then it will not have a significant impact on urine N dynamics or N leaching.

- Nitrous oxide emissions were reduced in spring 2017 by plantain swards compared to ryegrass-white clover swards. However, in autumn/winter of 2018, N_2O emissions were greater from plantain swards than ryegrass-white clover swards due to wetter soil conditions under plantain. This is a useful reminder that there are other soil factors that also influence N_2O emissions from soils. In autumn/winter, the urine from cows fed on plantain swards had a lower N concentration and so reduced N_2O emissions compared to the urine from cows fed on ryegrass-white clover swards.

- Ammonia losses were affected by plantain urine. In spring and autumn/winter, NH_3 losses from urine of cows fed plantain were lower than from urine of cows grazing ryegrass-white clover due to urea-N in urine being half that of urine from cows fed on ryegrass-white clover.

- Aucubin applied at 10 mg g^{-1} DM of plantain reduced N_2O emissions from ryegrass-white clover by 36%.

- Plantain urine did not reduce N_2O emissions compared to ryegrass-white urine, when both urine with the same N loading rate were applied to a ryegrass-white clover sward.

6.5 REFERENCES

Christensen, C., Hedley, M. J., Hanly, J. A., & Horne, D. J. (2018). Duration-controlled grazing of dairy cows. 2: nitrogen losses in sub-surface drainage water and surface runoff. *New Zealand Journal of Agricultural Research, 62*, 48-68.

Dietz, M., Machill, S., Hoffmann, H. C., & Schmidtke, K. (2013). Inhibitory effects of *Plantago lanceolata* L. on soil N mineralization. *Plant and Soil, 368*(1-2), 445-458.

Gardiner, C. A., Clough, T. J., Cameron, K. C., Di, H. J., Edwards, G. R., & de Klein, C. A. M. (2017). Potential inhibition of urine patch nitrous oxide emissions by Plantago lanceolata and its metabolite aucubin. *New Zealand Journal of Agricultural Research*, *61*(4) 495-503.

Minnée, E. M. K., Leach, C. M. T., & Dalley, D. E. (2020). Substituting a pasture-based diet with plantain (Plantago lanceolata) reduces nitrogen excreted in urine from dairy cows in late lactation. *Livestock Science, 239*, 104093.

Shepherd, M., Carlson, B., & Lucci, G. (2017). The effect of time of winter urine deposition on mineral nitrogen leaching and implications for grazed forage crops. New Zealand Journal of Agricultural Research, *60*(4), 376-385.

Shepherd, M., Phillips, P., & Snow, V. (2011). The challenge of late summer urine patches in the Waikato region. In: Adding to the knowledge base for the nutrient manager. (Eds L.D. Currie and C L. Christensen). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 24. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. 8 pages.

Wheeler, D., Cichota, R., Snow, V. & Shepherd, M. (2011). A revised leaching model for OVERSEER® nutrient budgets. In: Adding to the knowledge base for the nutrient manager. (Eds L.D. Currie and C L. Christensen). http://flrc.massey.ac.nz/publications.html. Occasional Report No. 24. Fertilizer and Lime Research Centre, Massey University, Palmerston North, New Zealand. 6 pages.

APPENDIX 1:

ADJUSTMENT OF THE METHODOLOGY FOR N_2O MEASUREMENT

In April 2017, a field experiment was carried out to measure N_2O emissions after urine application, similar to the field experiments which were carried out in spring 2017 and autumn/winter 2018 (Chapter 4). In that study, it was observed that N_2O fluxes were not consistent with the treatments, and the increase in N_2O concentrations over an hour of sampling (0, 30 and 60 minutes) was not linear even when urine was applied (Data not shown). The N_2O chambers were sealed only with soil around the base of the chamber (Figure A1b), therefore, a more effective seal system was required. In August 2017, another field experiment was conducted to evaluate different ways to seal the chambers and to determine the best sampling period for the chambers.

The experiment was divided into two studies: a) carbon dioxide flux (CO_2) and b) N_2O re-absorption. The aim of the first study was to evaluate different ways to seal the chambers and determine the most effective where the CO_2 flux was linear over the measurement period. The objective of the N_2O re absorption study was to evaluate if the N_2O emitted from the 'urine patch' was re absorbed by the pasture surrounded it due to the area cover by the chamber was larger than the area covered by the urine patch.

a) Carbon dioxide flux

The experiment was carried out in the field and the three ways to seal the chambers were evaluated: i) chamber with metal ring plus a seal system which was the plastic wrap (MRW) (Figure A1a); ii) chamber seals only with soil (NR) (Figure A1b); and iii) chamber seals with metal ring (MR) (Figure A1c). Each treatment was replicated 4 times. Chambers were closed for two hours due to the large volume of the chambers, and gas samples were taken at 0, 60 and 120 minutes. Gas samples (25 mL) were collected from a sampling port on the chamber using a plastic syringe and injected through a septum into evacuated sample vials.

Figure A1. a) Chamber seal with metal ring and plastic wrap, b) chamber without ring, and c) chamber with metal ring.

b) Nitrous oxide re absorption

This experiment was a field experiment. Tokomaru soil was collected from Massey University's Dairy Farm 4, air-dried and sieved (2 mm). To simulate the urine patch, 300 ppm of nitrogen (N) as potassium nitrate (KNO_3) fertiliser was applied to 2.5 kg of soil and placed into a plastic tray of 40 cm diameter inside the chamber (Figure A2).

Two treatments were evaluated: i) sealed chamber with metal ring and bicycle tube (MRS) (Figure A2b), and ii) sealed chamber with metal ring and bicycle tube, and a plastic bag which covered the grass inside the chamber to avoid N_2O reabsorption (MRSP) (Figure A2a). Each treatment was replicated 4 times.

Figure A2. a) Chamber's floor is cover with a plastic bag; b) chamber's floor is uncovered. These chambers were sealed with a bicycle tubes, which were inflated to provide a gas-tight seal.

Gas samples (25 mL) were collected from a sampling port on the chamber using a plastic syringe and injected through a septum into evacuated sample vials. The sample period lasted two hours and N_2O samples were taken at 4 sampling times (0, 30, 60 and 120 minutes).

Gas samples were analysed using a gas chromatograph. The emissions over the sampling time were integrated over time for each chamber to estimate the total emissions during the measurement period.

RESULTS

a) Carbon dioxide flux

Figure A3 shows the CO_2 fluxes during the two hours that chambers were closed. Only the chambers that were sealed with the plastic wrap shows a linear increased of the CO_2 concentration over the two hours. Chambers sealed only with the metal ring or with soil, show that the CO_2 accumulation was not linear over time, indicating that those chambers were leaking gas. These results show that the chambers used to determine N_2O fluxes have to be sealed.

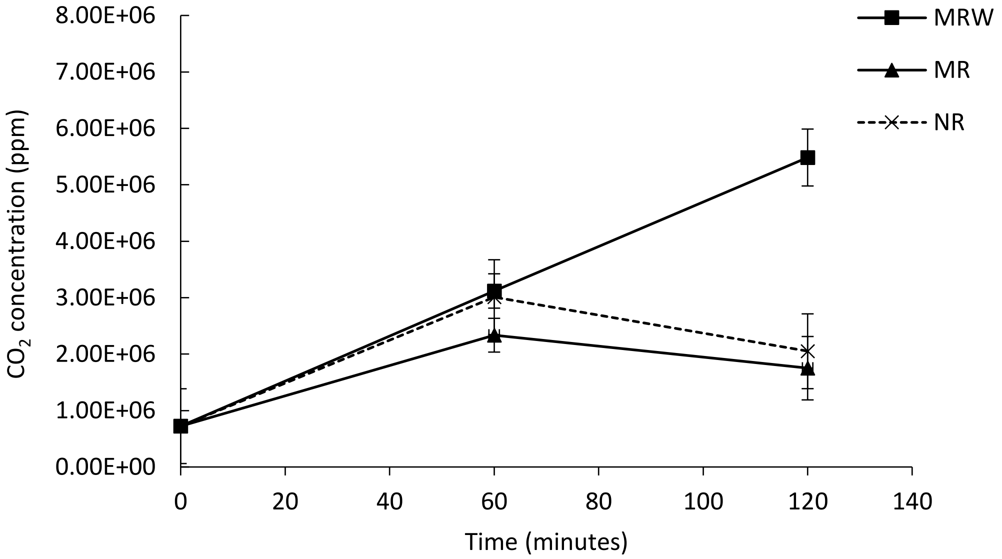


Figure A3. CO_2 fluxes during the two hours period.

Although the plastic wrap seals the chambers effectively, a more practical way to seal the chambers has to be used in the field experiment. Therefore, chambers will be sealed using bicycle tubes which will be inflated once the chambers will be placed inside the metal ring.

b) N_2O re- absorption

Average N_2O fluxes from both treatments (chambers sealed with bicycle tubes and chambers sealed with bicycle tubes plus the plastic bag covering the floor) were not significantly different (*P* > 0.05) (Figure A4). Therefore, this result shows that there is no reabsorption of N_2O by the edge where urine is not applied.

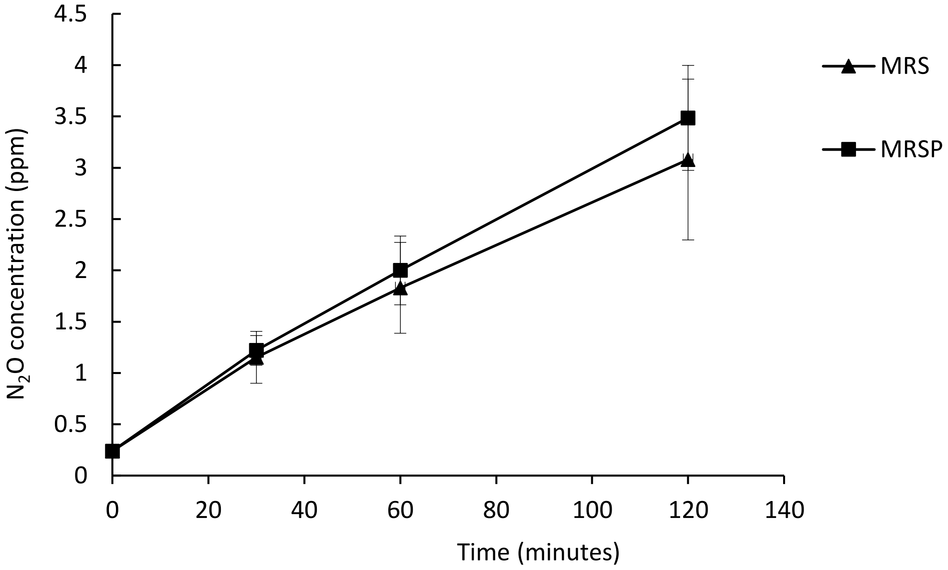


Figure A4. N_2O fluxes during the two hours period.